Novel control of the sheep scab mite, *Psoroptes ovis,* through the application of bacteriophage therapy.

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Abstract

Psoroptes ovis mites are the causative parasites of sheep scab disease. It is a contagious disease which causes intense pruritus, wool loss and the development of lesions. These lesions are exacerbated by secondary bacterial infections. Bacteria appear to play an integrated role in the pathogenicity of this disease and are found in the internal cavities of *P. ovis*. The aim of this study was to investigate these bacterial associations, with the aim of identifying a microbial target for sheep scab control. The microbial communities associated with sheep scab were investigated using both molecular and bacteriological techniques. Several environmental niches were targeted: scab-infected fleece, internal mite cavity and excreted faecal trails.

Microbial communities were very complex, with a variety of species and bacterial groups identified. Some bacteria were common to all environments, whereas others were isolated from one sample. Both natural and *in vivo* cultured mites were investigated in an attempt to identify universal and potentially beneficial bacteria. In addition, *P. ovis* mites were screened using PCR to detect potential endosymbiotic bacteria. Positive identification was made of *Comamonas* sp. in both natural and *in vivo* cultured mites; this species has been identified as an endosymbiont in other arthropods and its role in *P. ovis* requires further investigation. *In vitro* feeding experiments were carried out with *P. ovis* mites in the laboratory. Initially mite chambers were constructed and optimised to encourage maintenance of *P. ovis* off-host. A number of diets were tested and antibiotics were compared for their effect on bacteria within *P. ovis*. *In vitro* experiments revealed that *P. ovis* survival was significantly reduced with the administration of antibiotics and there was also evidence that they altered internal bacterial densities.

The potential of bacteriophage therapy for the microbial control of bacteria associated with *P. ovis* was investigated. Bacteria isolated from *P. ovis* faecal trails were used to isolate bacteriophage from environmental samples. Sixteen bacteriophage were successfully isolated, which were infective against three mite faecal bacteria. Isolated bacteriophage were characterised by a number of methods including their response to chemicals, enzyme and infection dynamics in both solid and liquid phases. *In vitro* experiments with bacteriophage were also investigated, resulting in a significantly reduced mite lifespan seen with some bacteriophage lysates. The potential for using bacteriophage for the control of *P. ovis* mites is discussed.

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Declaration

I hereby declare that the production of the thesis presented for examination for the degree of PhD, was completed by myself and the work presented herein is my own, except where explicitly stated otherwise in the text.

Sarah Hall

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Abbreviations

DVM	Divisional Veterinary Manager
cDNA	Complementary DNA
CFU	Colony Forming Unit
CI	Cytoplasmic Incompatibility
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
ELISA	Enzyme-Linked Immunosorbent Assay
FISH	Fluorescent In-situ Hybridisation
IgG	Immunoglobulins
ITS	Internal Transcribed Spacer
LB	Luria Bertani Broth
MFB	Mite Faecal Bacteria
MLs	Macrocyclic Lactones
MOI	Multiplicity of Infection
MRD	Maximum Recovery Diluent
MRS	de Man, Rogosa, Sharpe agar
NA	Nutrient Agar
NB	Nutrient Broth
OD	Optical Density
OP	Organophosphate
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PFU	Plaque Forming Units
PHI	Peterson's Homogeneity Index
PBS	Phosphate Buffered Solution
PSB	Bacteriophage Buffer
RE	Restriction Enzyme
R_f	Relative Mobility
RISA	Ribosomal Intergenic Spacer Analysis
rRNA	Ribosomal RNA
RT	Room temperature
SAC	Scottish Agricultural College
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SP	Synthetic Pyrethoid
SQ	Søerensen's Quotient
ssDNA	Single Stranded DNA
ssRNA	Single Stranded RNA
TEM	Transmission Electron Microscopy
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal –Restriction Fragment Length Polymorphism
ZOI	Zone of Inhibition

1 Background

1.1 Introduction

1.1.1 Sheep Scab Disease

Sheep scab is a potentially fatal disease in sheep caused by an infestation by the non-burrowing mite *Psoroptes ovis*. It is an important global disease, both for the welfare of the sheep and with regards to the financial outcome for the farmer (Baker & York, 1999). With 50 million sheep in the UK at risk from infection (Mathieson & Lehane, 1996), it has become endemic in hill and lowland areas of the British Isles (Oliveira *et al.*, 2006). In Scotland, an estimated 16.5% of flocks were infected in 2008 (ADAS UK Ltd, 2008). In addition to sheep, this disease is also known to infest goats, horses, camelids and rabbits (Bates, 1999a) with new incidences in cattle also reported recently (Mitchell, 2010). Rabbits are infested with *Psoroptes cuniculi,* which cause psoroptic otocariasis and scabs within the rabbit ears (Rafferty & Gray, 1987; Pettit *et al.*, 2000). *P. cuniculi* are thought to be variants of *P. ovis* adapted to aural environments (Wall & Shearer, 2001) but there is little molecular evidence that they are separate species (Pegler *et al.,* 2005).

In the UK in 2005, this disease was estimated to have an economic cost of over £8 million per annum (Nieuwhof & Bishop, 2005), which includes lost performance, prevention, and the most costly - treatment. Treatment of this disease is especially important if sheep are to be transported or sold, due to the potential transmission of infection to a number of other animals (Morgan-Davies *et al.*, 2006). In 2005, the Moredun Institute's National Sheep Health Survey declared that in Scotland the "greatest perceived threat to sheep health and welfare in the national flock was sheep scab" (Long, 2010).

Sheep scab has been eradicated from a number of countries including Australia (1884), New Zealand (1985), Canada (1973) and Great Britain (1952) (Kirkwood 1986; O'Brien 1999). It was reintroduced to the UK in 1973 *via* an infected import (ADAS UK Ltd, 2008) and in December 2010, sheep scab disease was re-instated as a notifiable disease under the Sheep Scab (Scotland) Order 2010. This requires sheep keepers to notify their local Divisional Veterinary Manager (DVM) if they have or know about, a case of sheep scab disease. The current UK prevalence of this disease is not accurately known (Bisdorff *et al.*, 2006), yet it is said to be endemic to the majority of the British Isles (ADAS UK Ltd, 2008). In a survey of Welsh farmers in 2010, 36.5% reported to having

scab in the last five years (Cross *et al.*, 2010). The recent legislation is likely to produce updated and more accurate figures of the prevalence of this disease.

1.1.2 Variations in the Disease

There are a number of factors thought to affect the prevalence and severity of sheep scab disease. Variables in the host animal, such as sheep breed, appear to affect the incidence due to wool type and composition which provides the habitat for the causative parasite, the sheep scab mite, *Psoroptes ovis* (Meyer & Neurand, 1991; Losson *et al.*, 1999; Fourie *et al.*, 2002). Breed differences in cattle have also been observed in the severity of this disease (Losson *et al.*, 1999). Different individual responses are observed both within a flock (Lewis, 1997) and also, whether it is a primary infestation or a secondary infestation in a sheep/flock with previous history of sheep scab (Bates, 1999b).

Spatial and temporal differences are known in prevalence (French *et al.*, 1999) and disease severity (Mathieson & Lehane, 1996; Bates, 1999b). Although historically reported as a winter disease, incidences are now reported throughout the year (Kirkwood, 1986; Lewis, 1997).

1.1.3 Sheep Scab Disease Clinical Signs

Animals infected with sheep scab disease have been shown to have reduced feeding- and resting times (Baker & York, 1999) and in some cases, the presence of mites cause sheep to display higher frequencies of abnormal behaviours, in response to irritation, such as 'nibble' reflexes, hyperaesthesia and even epileptic fits, which all have severe animal welfare consequences (Corke & Broom, 1999), sheep can also be observed kicking at their flanks and rubbing against structures such as fence posts (Scott, 2010).

The loss of fleece and development of lesions can be seen clearly in the later stages of the infection (Figure 1.1). Serum exudation discolours the skin and fleece giving it a moist yellow appearance (Scott, 2010). Eight weeks of infestation often result in hair loss of up to 20 cm on the flank, with hyperaemia (engorgement due to increased blood flow) and skin at the centre becoming keratinised (Scott, 2010).

Treatment of the disease requires accurate diagnosis, since similar symptoms may be observed with other ectoparasite infections (for example chewing lice) (Scott, 2010), especially for 'mildly, early and chronically affected' cases (Lewis, 1997). Currently, skin

scrapings and observation of live mites by microscopy are used by veterinary diagnostic laboratories (D. Henderson, *pers. comm;* Scott, 2010). New diagnostic tests, such as enzyme linked immunosorbent assays (ELISA) are being developed to enable earlier, and more accurate identification of sheep scab disease (Long, 2010).

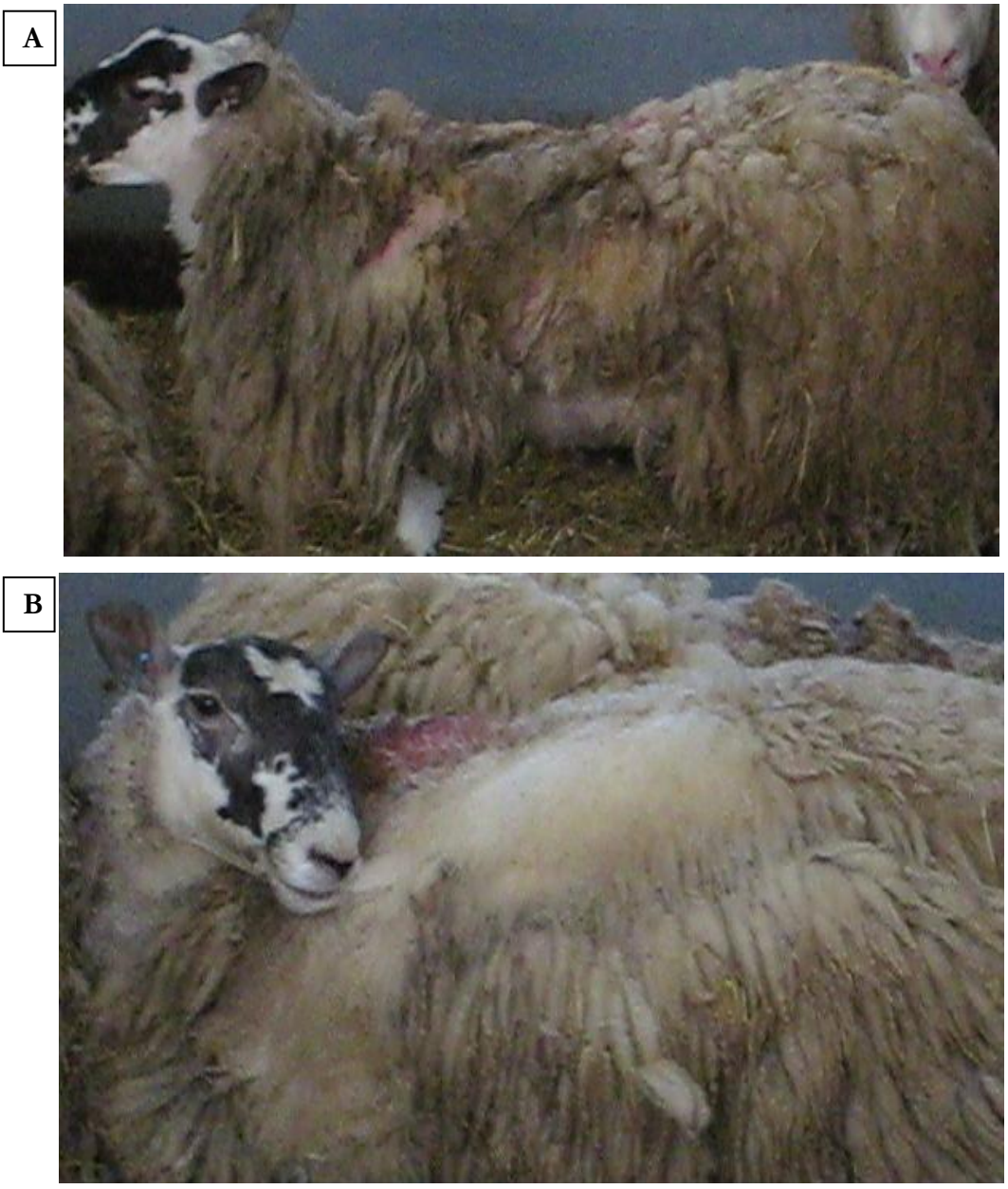

Figure 1.1 Clinical signs of fleece loss and lesions indicative of sheep suffering from scab disease (A). B shows a sheep pulling out its fleece.

1.1.4 Aetiology of Sheep Scab Disease

Sheep scab disease is caused by the non-burrowing mammalian ectoparasitic mite *Psoroptes ovis* (Acari: Psoroptidae) which lives on the skin at the base of the wool. Adult mites average 500 μm in length (Figure 1.2A). The mite causes extreme pruritus (severe itching), which leads to the development of lesions (Baker & York, 1999). Health and condition of the animal can deteriorate rapidly; large weight loss may be seen (Kirkwood, 1980) and if the animal is left untreated, the disease may also be fatal. Death is rare, but may occur as a result of debilitated animals suffering from wasting or in rare circumstances from anaphylactic shock (B Hosie, *pers. comm.*). It is thought the allergic inflammation and immune response that occurs on the sheep skin is caused by guanine and bacteria-rich mite faecal pellets deposited onto the skin (Lewis, 1997). Sheep scab is an extremely contagious disease (Arlian *et al.*, 1981; O'Brien *et al.*, 1994b) and spreads by direct contact with other hosts (Baker & York, 1999). *P. ovis* mites have been shown to remain infective off-host for up to 16 days and sites where infected animals have been, such as auctions and transporting vehicles, may be sources of new infection for other sheep (Sargison *et al.*, 1995).

1.1.5 *P. ovis* Feeding and Digestion

The exact diet of *P. ovis* is unknown. Historically it was thought that *P. ovis* pierced the skin of sheep (Kirkwood, 1986). Ultrastructural evidence, however, now indicates that *P. ovis* feeds mainly on debris on the epidermis produced as a result of the sheep scratching; notably serous exudates and lipids (Sinclair & Filan, 1989; Mapstone *et al.*, 2002). Observations of *P. ovis* mouthpart ultrastructures revealed grooves along which liquid food moves prior to being ingested along a food canal (Blake *et al.*, 1978; Mapstone *et al.*, 2002) (Figure 1.2B). *P. ovis* mites are then thought to abrade the loose stratum corneum (outer epidermis layer) (Kirkwood, 1983; Sinclair & Filan, 1989; Mathieson & Lehane, 1996). It is the remaining exudates that, on drying and crusting, cause the scab lesions (Baker & York, 1999). The mites are also noted to ingest bacteria on the skin surface (Sinclair & Filan, 1989), which may be either of mite or environmental origin. These bacteria have also been shown to colonise the lesion wounds opportunistically, resulting in the sheep developing injurious secondary infections (Sinclair & Filan, 1989). The morphology of mouthparts appears to be

comparable between *P. ovis* and *P. cuniculi*, with no distinct differences between males and females in either species (Rafferty & Gray, 1987).

P. ovis, as well as other closely related arthropods, have been shown to digest host immunological components such as immunoglobulins (IgGs), complement and cells (Willadsen, 1997), suggesting that they contribute to the mites' diet (Pettit *et al.*, 2000). Additionally, in ectoparasitic arthropods, proteinases are the major form of digestive enzymes, involving hydrolysis by cysteine and asparytl proteases. A detailed study of the characterisation of digestive enzymes of *P. cuniculi* has been carried out by Nisbet and Billingsley (1999b). They detected a number of different enzymes including phosphatases, esterases, leucine and valine aminopeptidases but did not detect any lipase activity. Feeding experiments have determined that *P. ovis* can ingest blood cells but prefer serum, plasma and water (Deloach, 1984; Sinclair & Filan, 1989). This lack of precise knowledge of the mite diet has contributed to a continued inability to maintain these mites *in vitro*.

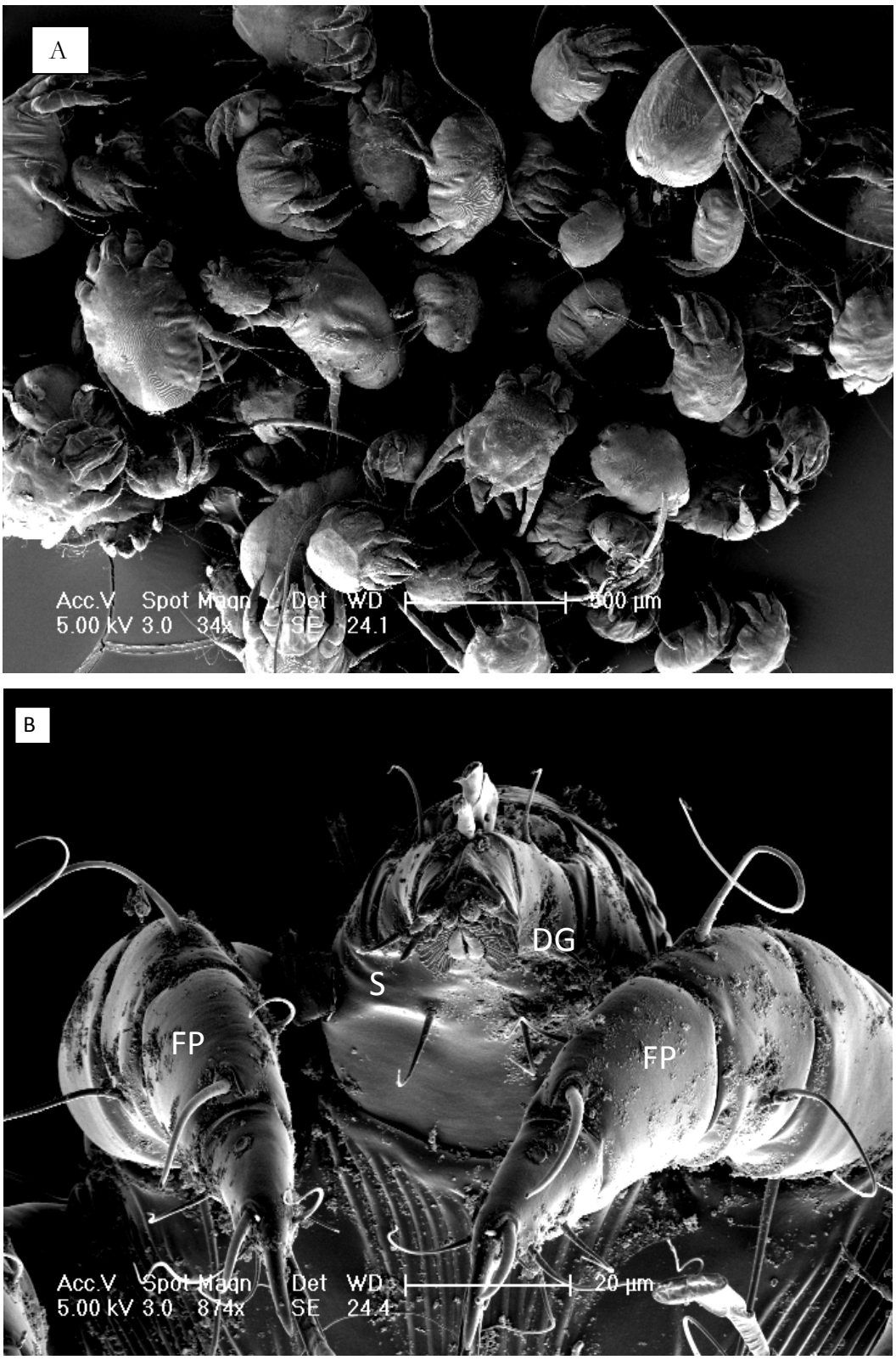

Figure 1.2 Scanning electron micrographs of *P. ovis* mites. **A**: A number of *P. ovis* individuals. **B:** Distal view of gnathosoma (DG) and first pair of pretarsi (FP) and sensillae (S) (Mapstone *et al.*, 2002). Separate scale bar for each. Images produced with assistance from M. Kierans, Microscopy Facility, University of Dundee, 2008.

1.1.6 *P. ovis* Lifecycle

Psoroptes ovis mites spend their entire lifecycle on the sheep host (Figure 1.3). Reproduction and female longevity are linked (inversely) and both are affected by atmospheric temperature (Downing, 1936). The key distinguishing feature of the *Psoroptes* genera is the presence of a relatively long, jointed pretarsus carrying a trumpet-shaped terminal pulvillus (sucker) on legs 1, 2 and 4 in adult females and legs 1, 2 and 3 in adult males (Wall & Kolbe, 2006).

Adult males have the distinguishing structure of anal suckers which are used to attach to the female tritonymph's rounded copulatory appendages (Figure 1.4). Copulation then takes place once the tritonmyph moults into the adult female (Baker & York, 1999).

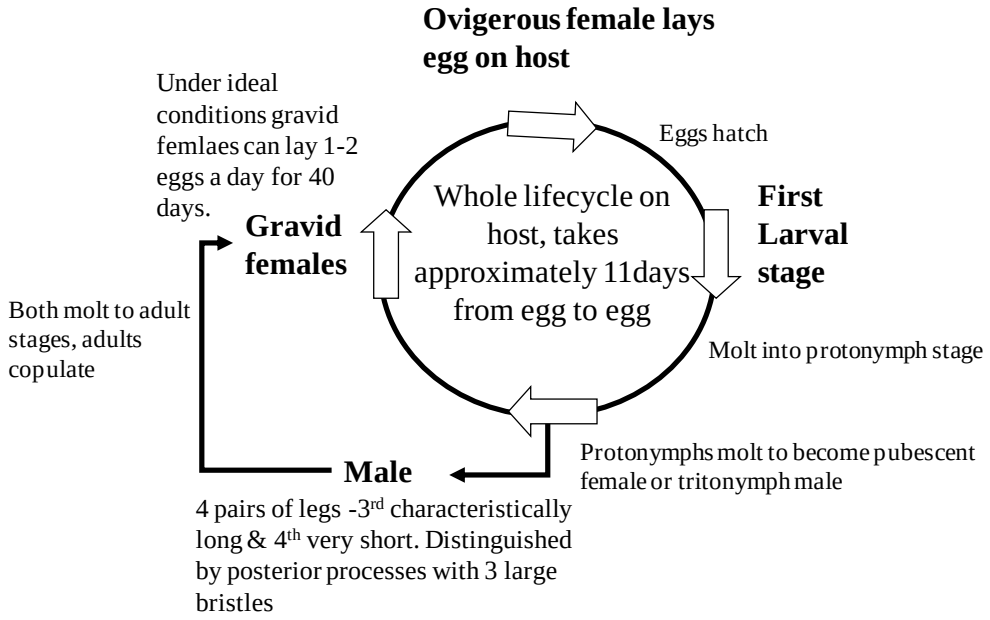

Figure 1.3 Lifecycle of *P. ovis* mites. Summarised from Downing 1936 and Baker & York 1999.

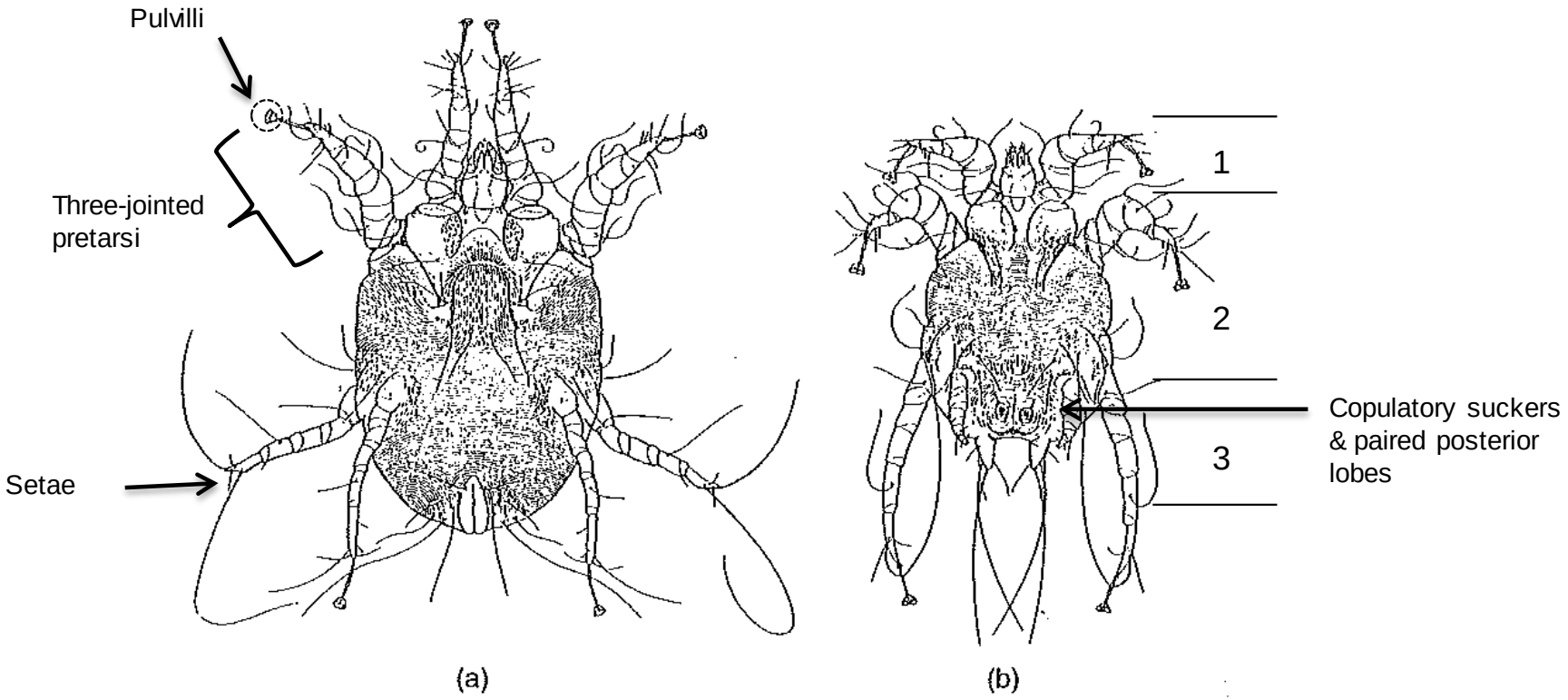

Figure 1.4 Differences in anatomy of male and female *P. ovis* adult mites *:* Adult female (a) ventral view, recognised by jointed pretarsi and pulvilli on first, second and fourth pairs of legs and setae on the third pair. Male (b) dorsal view, recognised by copulatory suckers and paired posterior lobes. Also have pulvilli on first three pairs of legs and setae on the fourth pair. Reproduced from Wall & Shearer (2001). Body can be divided into gnathsoma (1), podosoma (2) and opisthosoma (3) (Sanders *et al.*, 2000).

1.1.7 Sheep Scab Disease Pathology

Sheep scab disease has been characterised to have four main phases (Bates, 1997) (Figure 1.5)

i. Low mite numbers and small lesions may be seen.

ii. A rapid increase in mite numbers causing the lesion size to increase. Below the scab, circulating immunoglobulins (IgG) can be detected. As body heat dries the scab, the mites are forced to the periphery, where there is still fluid to ingest. The scab lesion is usually confined to the dorsal and lumbar areas of the sheep as hairy parts are unfavourable. Some animals affected at this stage are referred to as 'Flaker' sheep, characterised by cornflake-like scabs that lift to reveal thousands of mites (Bates, 1997). This is the most contagious time for transmission to other sheep and coincides with the peak oviposition stage for female mites.

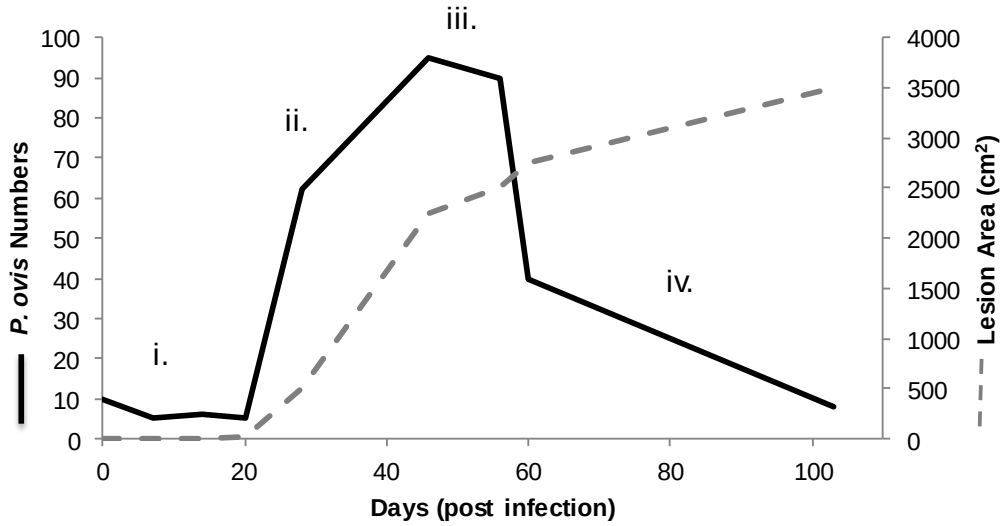

Figure 1.5 Disease pathology of Sheep Scab disease as shown by mite numbers and area of lesion (cm^2). Summarised from Bates (1997).

iii. A plateau phase is reached where mite numbers remain stable but lesion area and IgG titres continue to increase in response to skin irritation by dried mite faecal pellets and bacteria.

iv. There is a decline in mite numbers as the growth of the lesion slows and changes in characteristics; this results in the mite population also slowing due to

lack of feeding sites. The mites' fecundity may also be affected by the sheep immune response (Bates, 1997).

At the end of the above disease cycle, some sheep may recover from the infection as the mite population dies out. 'Pseudorecovery', however, may occur where mites move to cryptic sites, for example on the underside of the sheep, in the external auditory canal, inguinal folds (wrinkled scrotum of castrated males) and even on the tail of long-tailed sheep (Downing, 1936). Mites can persist asymptomatically in these sites for up to two years (Wall *et al.*, 1999). It is not clear what the mites feed on at this stage or whether they are in a phase akin to dormancy.

1.1.8 Disease Dynamics

It is important and necessary to understand the ecology of the mite if it is to be targeted successfully for control. Mathematical models have been produced to simulate the population dynamics of mites on a single sheep throughout an infection (Wall *et al.*, 1999) and also, of transmission between flock individuals (Berriatua *et al.*, 1999). Mites exhibit density independent growth that can increase by up to 11% per day, resulting in the population doubling every 6.3 days (Wall *et al.*, 1999). Since a necessary mortality level of 52% per two-day-period is required to halt the population growth (Wall *et al.*, 1999) this is important information regarding the design and execution of mite-control programmes.

1.2 Current Controls of Sheep Scab Disease

1.2.1 History

Sheep scab disease is one of the oldest known sheep diseases (Downing, 1936), with UK legislative control first initiated in 1798 (Table 1.1) (Kirkwood, 1986; O'Brien, 1999).

Table 1.1 Timeline of events in the history of sheep scab disease.

Date	Event
1798	Legislative control in UK to protect pastures from scab infected sheep.
1809	*Psoroptes ovis* identified as cause of Sheep Scab.
1843	Commercial dips introduced.
1869	Sheep Scab became a notifiable disease.
1905	Compulsory dipping introduced.
1948	Gamma Benzene Hexachloride (HCH) discovered. Advantage over other products as it remained acaricidal for many weeks after dipping.
1952	Sheep Scab eradicated from the UK.
1973	Sheep scab accidentally reintroduced to UK.
1976	Control practices (dipping) reinstated.
1986	Sheep Scab Order passed, which provides guidelines on treatment of infected sheep.
1992	Lifting of compulsory dipping in the UK.
2008	ADAS reviewed control and management of the disease (ADAS Ltd 2008).
2010	Sheep Scab (Scotland) Order 2010 passed, disease re-designated a notifiable disease.

1.2.2 Present Controls

There is a wide source of literature advising on the prevention and treatment of sheep scab (Downing, 1936; Coles *et al.*, 1994; Bates, 2004; Sargison, 2004; Hosie, 2005; Bates, 2007; Scottish Sheep Scab Initative, 2008; SCOPS, 2008). The ability of modern transport to move large numbers of sheep quickly is thought to have contributed to the spread and persistence of this parasite (Kirkwood, 1986), particularly since rediscovery in the UK after 1973.

There are three classes of compounds used currently to treat Sheep Scab disease; organophosphate compounds (OP's), synthetic pyrethroids (SP's) and macrocyclic

lactones (ML's) (Lewis, 1997) and they are all applied either in a plunge dips or as an injectable. There have been reported cases of resistance to all except the macrocylic lactone products (Lewis, 1997). All products must be licensed as 'scab approved', whereby they are "able consistently to produce a 100% kill of all mites in fully fleeced sheep, single plunge dip for one minute" (Hosie, 2005). In addition, protection has to be provided against re-infestation for 21 days or longer in sheep with a fleece length of 1 cm (Lewis, 1997). *P. ovis* mites can remain infective off the host sheep for up to 16 days and therefore, disease control approaches should also target other sources of infection, such as fence posts (which they scratch against), vehicles and even clothing.

1.2.2.1 Plunge dipping

Plunge dipping, compared to shower applications, is still regarded as the most efficient form of application of microemulsion treatments, since the complete submersion of the sheep helps to displace the air trapped within the fleece, ensuring treatment reaches the skin surface. It utilises very broad spectrum treatments, such as those containing the chemicals diazinon or high cis cypermethrin, that have the potential to also kill other ectoparasites such as keds and lice. Recently, however, cypermethrin dips were withdrawn by manufacturers after pollution and environmental safety concerns (Mitchell, 2010). In June 2008, however, it was estimated that only 20% of Scottish farmers were using this method (ADAS UK Ltd, 2008), the other 80% favouring injectables (Section 1.2.2.2). Low utilisation of this technique may be due to associated regulations, as currently a licence is required to dip (Hosie, 2005).

Dipping also has some associated problems. For example, in addition to resistance, the process is stressful for the sheep and labour intensive (Bates, 1993). There is also the direct environmental implications of waste disposal of toxic OPs (Groundwater regulations 1998) (ADAS UK Ltd, 2008) and there have been reports of sickness related to using OP dips by farm workers (Stephens *et al.,* 1995). Moreover, dipping is described as an 'exact science' (Bates, 2004), as concentrations need to be calculated correctly at the start and replenished throughout the process, which can provide logistical problems. There is also the problem of stripping, where the active ingredient of the dips is absorbed into the wool, depleting the concentration. Finally, administration of sub-lethal doses, which can lead to resistance, must be strictly avoided (Bates, 2004).

1.2.2.2 Injectables

Injectables, such as the avermectins, ivermectin, doramectin and also moxidectin, can be used for both prevention and the treatment of an occurring infection but unlike dipping products, are very specific to scab infections only (Hosie, 2005). Different products require a different number of injections for effectiveness (SCOPS, 2008) and accurate dosage depends on individual sheep weight (Lewis, 1997). Compared with the low percentage of dipping, 80% of Scottish farmers reportedly used injectables in 2008 (ADAS UK Ltd, 2008). One disadvantage with using this method is that the lesions associated with sheep scab are not bathed as in dipping and therefore, wounds may take longer to heal as the allergens are not washed away (Bates, 2004). The resistance range of some products has also increased as a result of their additional use on cattle to treat scab (Rehbein *et al.*, 2002). In addition to the problems listed above, there are issues with international sale regulations, meat withdrawal periods and side affects such as damaged fleece (O'Brien, 1999). The failures and limitations of the current controls to treat sheep scab disease results in the critical need for an alternative method of control.

1.2.3 Potential Alternative Control Methods

Alternative methods of sheep scab control that have been investigated include vaccines, entomopathic fungi, genetic modification of bacteria and breeding sheep for resistance.

Vaccination utilises the inflammatory secretions of the sheep immune system in response to allergens (Nisbet & Billingsley, 1999a), which have also been identified from both house-dust and storage mites (Pruett, 1999) and scab mites (Kenyon & Knox, 2002). Recently, sheep scab vaccine candidates have been identified by immunoscreening, which screens proteins of mite-origin against sheep immunoglobulins (IgGs) to construct a cDNA library to identify potential recombinant vaccine candidates (Nisbet *et al.*, 2008). Previous results have shown that sheep immunised with soluble mite extracts showed slower lesion growth and had fewer mites compared with controls (Smith & Pettit, 2004). Mite digestive enzymes have also been investigated as candidate compounds for immunological control of mites, which initiates the host animal's immune system to parasitic allergens (Nisbet & Billingsley, 1999b) further enhancing the design of control agents (Hamilton *et al.*, 2003).

Other biological controls that have been investigated include bacteria which produce harmful toxins, notably *Bacillus thuringiensis,* which produces the toxin thuringiensin

(Pinnock, 1994) and entomopathic fungi, particularly *Beaveria bassiana* (Taylor *et al.*, 2008) and *Metarhizium anisopliae* (Abolins *et al.*, 2007) both of which have been shown to be toxic to mites. Different sheep breeds appear to show different levels of susceptibility to this disease (Smith *et al.*, 2001), although breeding programmes to counteract this would be complicated by the ultimate desired trait and commercial value of the sheep, whether for meat or wool production. Recently trans-cinnamic acid, an essential oil, has been investigated for its suitability to control *P. ovis* (Wall & Bates, 2011). The results were extremely promising with 95% mortality of mites observed with a concentration as low as 6.29% (v/v) of the trans-cinnamic acid.

One approach that has been suggested as a potential control approach involves targeting potential endosymbiont bacteria which have a specific association with mites and a key role in survival and/or reproduction (Douglas, 1989). The following review examines bacterial associations in arthropods.

1.3 Bacterial Associations in Arthropods

1.3.1 Endosymbiont Introduction

Many organisms have symbiotic relationships. This symbiosis may be; mutualistic, beneficial to both organisms, commensal, where one benefits yet the other is not harmed or parasitic, in which one organism benefits to the detriment of the other. If one organism lives within the other, it is referred to as endosymbiosis, yet the effects of their presence can still be mutualistic, commensal or parasitic. This thesis is primarily concerned with the presence of bacteria that reside within *P. ovis* mites and irrespective of their function or location within mites, will hence be referred to as endosymbionts.

Obligate (primary) endosymbionts are organisms which have formed intrinsic relationships with hosts that are essential to both host and bacterium. These types of endosymbiont often cannot be cultured outside of the host (Wernegreen, 2004). Obligate endosymbionts are most commonly transmitted vertically from mother to offspring (Zchori-Fein & Perlman, 2004). For example, tsetse fly larva receive two gut endosymbionts from its female parent *via* milk-gland secretions, whereas a third, reproductive endosymbiont, is transovarially transmitted (Aksoy, 2000). Alternatively, they may be facultative or secondary endosymbionts. These are not always present in every individual within the population and may be transmitted vertically or horizontally between members in the population (Wernegreen, 2004). Within their specific internal

distributions, bacteria may be found immersed within the cytoplasm, for example *Cardinium* (Nakamura *et al.*, 2009) or located within vacuoles, separated from the cytoplasm by a membrane, for example *Wolbachia* (Zouache *et al.*, 2009b).

Endosymbionts are known to play a central role in the survival, development, ecology and evolution of their arthropod hosts (Brand *et al.*, 1975; Brune, 2003). Loss of these microbes can often result in abnormal development and reduced survival or pathogen resistance of the arthropod host (Eutick *et al.*, 1978b; Fukatsu & Hosokawa, 2002). They may be extracellular, in the digestive tract lumen, or intracellular in specialised organelles (Douglas, 1989).

Specifically, these endosymbiont bacteria may provide better nutrition (Buchner, 1965), heat tolerance (Dunbar *et al.*, 2007) and pathogen resistance (Hedges *et al.*, 2008; Zouache *et al.*, 2009b) or be pathogenic (Min & Benzer, 1997) and exhibit negative reproductive effects on the host (Zchori-Fein *et al.*, 2001; Zouache *et al.*, 2009a). Their effects can also be dependent on environmental factors (Duron *et al.*, 2008). Beneficial bacteria are commonly found in arthropods living on specialised, and often narrow diets, and have been shown to synthesise essential nutrients and cofactors for the host (Atlas & Bartha, 1993; Egert *et al.*, 2003; Banjo *et al.*, 2005).

Endosymbionts may live within the body of the host without causing any negative effects on the host. *Buchnera aphidicola* for example, provides amino acids to their aphid host (Baumann, 1997) and gut protozoa in termites ferment otherwise indigestible cellulose (Baumann *et al.*, 1997). Blood-feeding hosts often harbour endosymbiotic bacteria for example, the tsetse fly (G*lossina morsitans*) and its primary endosymbiont *Wigglesworthia glossinidia,* which provides B vitamins otherwise absent from the host's mammalian blood diet (Aksoy, 1995; Wernegreen, 2004). In addition to nutrition, endosymbiotic bacteria also play crucial roles in the reproduction of arthropods, such as normal egg development (Son *et al.*, 2008). Microbial endosymbionts may also convey signals between hosts; house flies (*Musca domestica)* cover eggs with the bacterium *Klebsiella,* this bacterium initially acts as an oviposition stimulant to other flies but after a proliferation threshold is reached, it inhibits oviposition by conspecific flies to assist with egg success potential (Lam *et al.*, 2007). Endosymbionts can also have detrimental effects on host reproduction, including parthenogenesis, feminisation and cytoplasmic incompatibility (CI) (Zchori-Fein & Perlman, 2004). CI is the most common effect of

Wolbachia infection (Gotoh *et al.*, 2007; Zouache *et al.*, 2009b). Endosymbionts have also been observed to have pathogenic effects on life history (Grimont & Grimont, 1978).

There appears to be a link between distribution of the endosymbiotic bacterium within the host and the specific role of the bacterium. Some endosymbionts are located in a single organ; *Cardinium,* for example, has only been found to date in the reproductive organs of the cicadelid, *Scaphoideus titanus* (Bigliardi *et al.*, 2006). This bacterium causes parthenogenesis in the parasitoid host, *Plagiomerus diaspidis* (Matalon *et al.*, 2007). In Flat mites (*Brevipalpus* sp.*)*, however, *Cardinium* is found throughout the body yet is thought to have a role in feminisation in this species. Its levels vary between individuals, instars and inner organs (Kitajima *et al.*, 2007). In the predatory mite *Metaseiulus occidentalis*, *Cardinium* has been shown to have positive effects on mite fecundity, although its internal location was not determined (Weeks & Stouthamer, 2004).

There has been a wealth of research into the effects on hosts of selectively removing endosymbionts. The most common method is through the use of antibiotics, which may be administered through a number of routes. For example, antibiotics may be administered in diets, such as used for the pea aphid, *Acyrthosiphon pisum* (Wilkinson, 1998) or vine weevils, within the family Curculionidae (Son *et al.*, 2008). They may also be injected directly into the body cavity as used for the tsetse fly (*G. morsitans*) (Nogge & Gerresheim, 1982). Douglas (1989) suggested the potential for pest control through disruption of their endosymbionts. Bacteriophage, viruses of bacteria, have been proposed as a novel method of selectively removing arthropod symbionts (Blackwell & Wellburn, 2003).

1.3.2 Potential Sheep Scab Mite Targets for Development of Novel Control Mechanisms

Opportunistic bacteria on sheep skin may infect the wounds that are characteristic of sheep scab disease. These bacteria may be of environmental origin but potentially also from the mite. Bacteria have been observed in the gut of *P. ovis* mites (Mathieson, 1995) and are thought to be delivered to the sheep's skin *via* mite faecal pellets (Mathieson & Lehane, 1996).

1.3.2.1 Bacteria of Sheep and Environmental Origin

A number of studies have investigated the bacteria associated with sheep diseases and mites (Merritt & Watts, 1978b; Murray & Edwards, 1987). Many of the bacterial species are ubiquitous and commonly found in the environment (Table 1.2) and healthy sheep

have an abundance of such bacteria naturally resident on the fleece. Typical bacterial species identified as resident on sheep fleece include *Bacillus cereus* (Bates, 2003) and *Staphylococcus aureus* (Oliveira *et al.*, 2006).

The microbial community of the fleece, however, is dynamic and may become dominated by different species depending on exposure to different environmental conditions (London & Griffith, 1984). For example, wetting of fleece may result in fleece rot which will alter the population dynamics of skin-fleece bacteria often allowing opportunistic pseudomonads to exhibit superior survival and growth compared to other bacterial species (Chin & Watts, 1992). In addition, *S. aureus,* another common skin bacteria, may be a source of 'potent superantigens' (Oliveira *et al.*, 2006), which may add to the severity of the wound infections.

1.3.2.2 Bacteria of Mite Origin

Previous work has isolated a number of bacteria from *P. ovis* mites, including *Serratia marcescens*, *Alloiococcus otitidis*, *Curacaobacter baltica*, *Pantoea agglomerans*, *Phyllobacterium rubiacearum* and *Proponibacterium acnes* as well as other unidentified species (Table 1.2) (Hogg & Lehane, 1999; Hogg & Lehane, 2001).

Generally, it is not clear what role these bacteria play in the association with the mite, but Hogg & Lehane (2001) noted many species present were characterised by their ability to colonise arthropod midguts and production of extracellular lipase. Some genera, notably *Serratia,* have been noted previously to exhibit pathogenic effects on the arthropod host, especially when ingested in large quantities (Grimont & Grimont, 1978).

The studies reviewed (Table 1.2) also found variations in the skin microflora of sheep due to different moisture and protein levels (Merritt & Watts, 1978a), pH of the skin (Meyer & Neurand, 1991) and also, the food consumed by the host (Lee *et al.*, 2008). Different sheep breeds provide different environments for bacteria. For example, wool-producing animals with long and fine high quality fleece, such as Merinos, offer microbes protection by their dense interweaving wool fibres and favourable nutritional status associated with the high organic contents of wool grease (Meyer *et al.*, 2001). Bacteria associated with sheep scab may also be a potential source of toxins that increase the pathogenicity of the disease. House dust mites (*Dermatophagoides pteronyssinus*) have been shown to carry *Bartonella* among other Gram negative bacterial species, all of

which have been suggested to be the source of endotoxins found in the mite allergenic extracts (Valerio *et al.*, 2005).

Table 1.2 Bacteria associated with *P. ovis* mites and associated arthropods. Continued on page 21

Phylum	Bacteria species	Origin	Reference
Acinetobacter	*Acinetobacter species (Table IV)*	House dust mite *(Dermatophagoides farinae)*	Valerio *et al* 2005
Actinobacteria	*Bifidobacteriaceae*	Bee mite *(Varroa destructor)*	Nisbet & Blackwell 2009
	C. baltica-related bacterium	*P. ovis* (midgut)	Hamilton *et al* 2003
	Corynebacterium spp.	*P. ovis* (midgut)	Hamilton *et al* 2003
	Propionibacteium acnes	*P. ovis* (midgut)	Hogg & Lehane 2001
Alpha-proteobacteria	*Bartonella* spp.	*Dermatophagoides pteronyssinus & D. farinae*	Valerio *et al* 2005
	Bradyrhizobium japonicum	*P. ovis* (midgut)	Hamilton *et al* 2003
	Wolbachia	Predatory mite (*Metaseiulus occidentalis)* , Mosquito (*Aedes albopictus*)	Hoy & Jeyaprakash 2005, Zouache *et al* 2009
	Phyllobacterium rubiacearum,	*P. ovis* (midgut)	Hogg & Lehane 2001
	Curacaobacter baltica	*P. ovis*	Hogg & Lehane 2001
Beta-proteobacteria	*Acidovorax*	Bee mite *(V. destructor),* earthworms	Nisbet & Blackwell 2009
	Burkholderia sp	*P. ovis (midgut),* Sandfly *(Lutzomyia longipalpis)*	Hamilton *et al* 2003, Gouveia *et al* 2008
	Comamonas	Bee mite *(V. destructor), mosquito (Ae. albopictus)*	Nisbet & Blackwell 2009, Zouache et al 2009
	Cupriavidus	Bee mite *(V. destructor)*	Nisbet & Blackwell 2009

Table 1.2 continued.

Phylum	Bacteria species	Origin	Reference
	Wigglesworthia	Tsetse flies (*Glossinidae glossinidia*)	Aksoy 1994, 1995, Baumann & Moran 1997
	Acinetobacter	Bee mite *(V. destructor), mosquito (Ae. albopictus)*	Nisbet & Blackwell 2009, Zouache *et al* 2009
	Escherichia coli,	House dust mite *(D. farinae)*	Valerio *et al* 2005
	Pantoea agglomerans	*P. ovis* (midgut), Mosquito *(Culex quinquefascinatus)*	Hogg & Lehane 2001, Pidiyar *et al* 2004
	Providencia stuartii	Chewing lice *(Damalinia ovis)*	Murray & Edwards 1987
Gamma Proteobacteria	*Pseudomonas stutzeri*	*D. ovis* lice, sheep fleece rot	Murray & Edwards 1987, London & Griffith 1984
	Rickettsiella	Red poultry mite *(Dermanyssus gallinae*), Bee mite (*V. destructor*)	De luna *et al* 2009, Moro *et al* 2009, Nisbet & Blackwell 2009
	Serratia marcescens	*P. ovis* (midgut), *P. cuniculi,* sheep fleece rot, Triatomine bugs (*Rhodnius prolixus)*	Chin & Watts 1992, Hogg & Lehane 1999, 2001, Mathieson & Lehane 1996, Azambuja 2004, Perrucci *et al* 2005,
	Stenotrophomonas	Bee mite *(V. destructor)*	Nisbet & Blackwell 2009
	unid pseudomonoads	*P. ovis* (midgut)*, D. farinae, Ae. albopictus*	Hogg & Lehane 2001, Valerio *et al* 2005, Zouache *et al* 2009
	Alloiococcus otitidis	*P. ovis* (midgut)	Hogg & Lehane 1999
	Bacillus spp	*P. ovis* (midgut), healthy sheep fleece	Murray & Edwards 1987, Chin & Watts 1992, Hogg & Lehane 2001, Oliveira et al 2006
	Micrococcus sp	*Damalinia ovis* lice	Murray & Edwards 1987
	Salinicoccus roseus	*P. ovis* (midgut)	Hamilton *et al* 2003
Firmicutes	*Staphylococcus intermedius/chromogens*	*P. ovis* (midgut)	Hogg & Lehane 1999
	Staphylococcus aureus	*P. ovis* mite (midgut), healthy sheep fleece, lice, (*D. ovis*) red poultry mite (*D. gallinae*)	Kloos et al 1976, Murray & Edwards 1987, Chin & Watts 1992, Hogg & Lehane 1999, Hogg & Lehane 2001, Moro *et al* 2009
	unid streptococci	*P. ovis*	Hogg & Lehane 2001

1.3.2.3 Potential Nutritional Bacterial Endosymbionts of *P. ovis*

As a result of their suspected narrow diets (Section 1.1.5) (Sinclair & Filan, 1989; Mathieson & Lehane, 1996), *P. ovis* mites may have close associations with beneficial bacteria. It is likely that they ingest bacteria from environmental sources, such as *B. cereus* and this association may be transitory, although knowledge of other bacterial species which may be present is limited. Uncharacterised endosymbionts from tick species have been detected in house dust mites (*Dermatophagoides farinae*) (Valerio *et al.,* 2005) and other host associations in closely related mite species include *Rickettsiella and Spiroplasma* found in red poultry mites, *Dermanyssus gallinae* (Moro *et al.*, 2009; De Luna *et al.*, 2009) and *Cardinium* which has been found in a wide distribution of arthropods, including predatory mites *Metaseiulus occidentalis* (Zchori-Fein & Perlman, 2004). These authors have suggested that these bacteria have roles in reproduction (*Spiroplasma*, *Cardinium*), as gut symbionts (*Cardinium*) and even be pathogenic (*Ricketsiella*). If it were possible to identify the bacterial communities associated with sheep scab mites, they could potentially be targeted to disrupt the lifecycle of the mites and so effect control of sheep scab disease.

The targeting of associated beneficial bacteria for the control of pathogens/arthropods has been demonstrated. This includes molecular genetic approaches to modify vector capability (Aksoy *et al.*, 2001) and the controlled expression of genes to arrest transmission of human pathogens by arthropods (Beard *et al.*, 1998). This method works either by expressing foreign anti-parasitic genes in symbiotic bacteria harboured by insects. Alternatively it may involve genetic manipulation of insects to confer refractoriness (inability to transmit pathogenic agents) to vector populations.

1.3.3 Detection Methods of Associated Bacteria

A large majority of bacteria associated with hosts are non-culturable by common microbiological methods, making their detection problematic (Baumann & Moran, 1997). For example, recreating the conditions of an arthropod gut *in vitro* would be almost impossible (Egert *et al.*, 2003) yet some success of culturing endosymbionts *in vitro* has been achieved. For example, the symbiont of *Triatoma infestans* has been cultured in *Aedes albopictus* cell lines (Hypsa & Dale, 1997). Culture-dependent techniques can give limited information on the diversity of bacteria associated with an organism. Additionally, there are a number of culture-independent methods including 16S rRNA

gene sequencing (Fox *et al.*, 1992; Higgins & Azad, 1995), ribosomal intergenic spacer analysis (RISA) (Cardinale *et al.*, 2004), fluorescence *in situ* hybridisation (FISH) (Janssen, 2006) and terminal restriction fragment length polymorphism (T-RFLPs) (Haynes *et al.*, 2003) which can aid in the characterisation of unculturable bacteria. These techniques are discussed in detail in Chapter 2. 16S rRNA is the small subunit of the cellular prokaryote ribosome that is responsible for protein manufacturing. This gene has been used to investigate the bacterial species associated with *P. ovis* (Hogg & Lehane, 1999; Hogg & Lehane, 2001). RISA utilises sequence heterogeneity in the internal transcribed spacer (ITS) region, non-coding DNA between the two functional 16S rRNA and 23S rRNA genes. Highly conserved regions in 16S and ITS are targeted by primers in which differences in the variable regions can be subsequently detected. These sequences can then be amplified by polymerase chain reaction (PCR) (Amann & Ludwig, 2000) followed by molecular sequencing to identify bacterial species. T-RFLPs use gene sequences and PCR methods in addition to restriction enzymes to produce 'fingerprints' of microbial communities.

Microbiological techniques such as biochemical tests and phenotypical methods, are good starting points for microbial diversity analysis. These techniques can be especially useful when the target bacterial species is known, as a number of selective methods can be used; such as CT agar for *Serratia* sp. (Starr *et al.*, 1976) or chromogenic agar for *Salmonella* detection (Gaillot *et al.*, 1999). Conversely, the use of reference tables for identification of bacterial groups/species or flowchart results (Cowan *et al.*, 2003) can be extremely powerful in preliminary bacterial classifications. There are, however, some intrinsic weaknesses with culture-dependent microbiological studies, a major concern being the inability to detect unculturable microorganisms. It is estimated that 99% of environmental bacteria are unculturable in the lab (Dillon & Dillon, 2004).

1.3.4 Biocontrol

Biological control (or biocontrol) utilises natural predators, parasites or pathogens of the host for pest management.

Arthropod pests or vectors of disease have been targeted with biocontrol. One example is the use of a virulent strain of *Wolbachia* which, when transmitted to aedine mosquitoes, resulted in a reduced lifespan of the host and subsequently reduced dengue fever transmission risks (Brownstein *et al.,* 2003). The reproductive manipulator features

of *Wolbachia*-induced cytoplasmic incompatibility (CI) have been investigated for the control of filiariasis-vector mosquito *Culex pipiens*, resulting in lack of, or infertile, offspring. This showed promise as a control method as female mosquitoes were unable to discern normal and incompatible males (Laven, 1967).

Bacteria have been observed internally within *P. ovis* (Mathieson & Lehane, 1996) and it is possible that beneficial internal relationships with bacteria may be present. Disruption of these bacteria may affect the longevity of *P. ovis*. One approach used to target bacterial infections is through the use of specific bacterial viruses, or bacteriophage. The following review examines the nature of bacteriophage and its use in targeting bacterial infections.

1.4 Bacteriophage

1.4.1 Introduction

Bacteriophage are viruses that infect bacteria. They are more numerous than bacteria and the 'most widely distributed and diverse entities' in the world (McGrath & van Sinderen., 2008) with approximately 10^{32} present on earth (MacGregor, 2008). Bacteriophage were first discovered in 1896 by Hankin in the River Ganges. He observed differential susceptibility to cholera linked to drinking river water but that protective property disappeared when the water was boiled (MacGregor, 2008). It was not until 1915 when Twort and subsequently D'Herelle in 1926 initiated research into bacteriophage (Levin & Bull, 1996). The popularity and use of bacteriophage declined, especially in Western countries, with the arrival of antibiotics (Levin & Bull, 1996). Recently, however, the 'impending crisis' of emerging antibiotic resistance in bacteria has resulted in the reassessment of bacteriophage therapy (Levin & Bull, 1996). There are also some institutes, such as the Institute of Immunology and Experimental Therapy in Poland that have continually utilised bacteriophage, especially for the treatment of multi-drug resistant bacterial infections, since 1926.

Currently, 599 species of bacteriophage have been identified; of these, 454 have dsDNA, 92 with ssDNA, 9 with dsRNA and 49 with ssRNA genomes although none have both DNA & RNA (Prangishvili, 2007) (Figure 1.6). Bacteriophage classification is 'open ended' with new species being identified regularly (Ackermann, 2009). Each contains a unique protein that protects particles from bacterial host nuclease, which

would otherwise break them down during an infection. Bacteriophage vary greatly in their structural complexity, ranging from a small icosahedral bacteriophage with a genome of 1-2 million Daltons to a large complex genome (e.g. T4) of 130 million Daltons (McGrath S & van Sinderen D, 2008) (Figure 1.7).

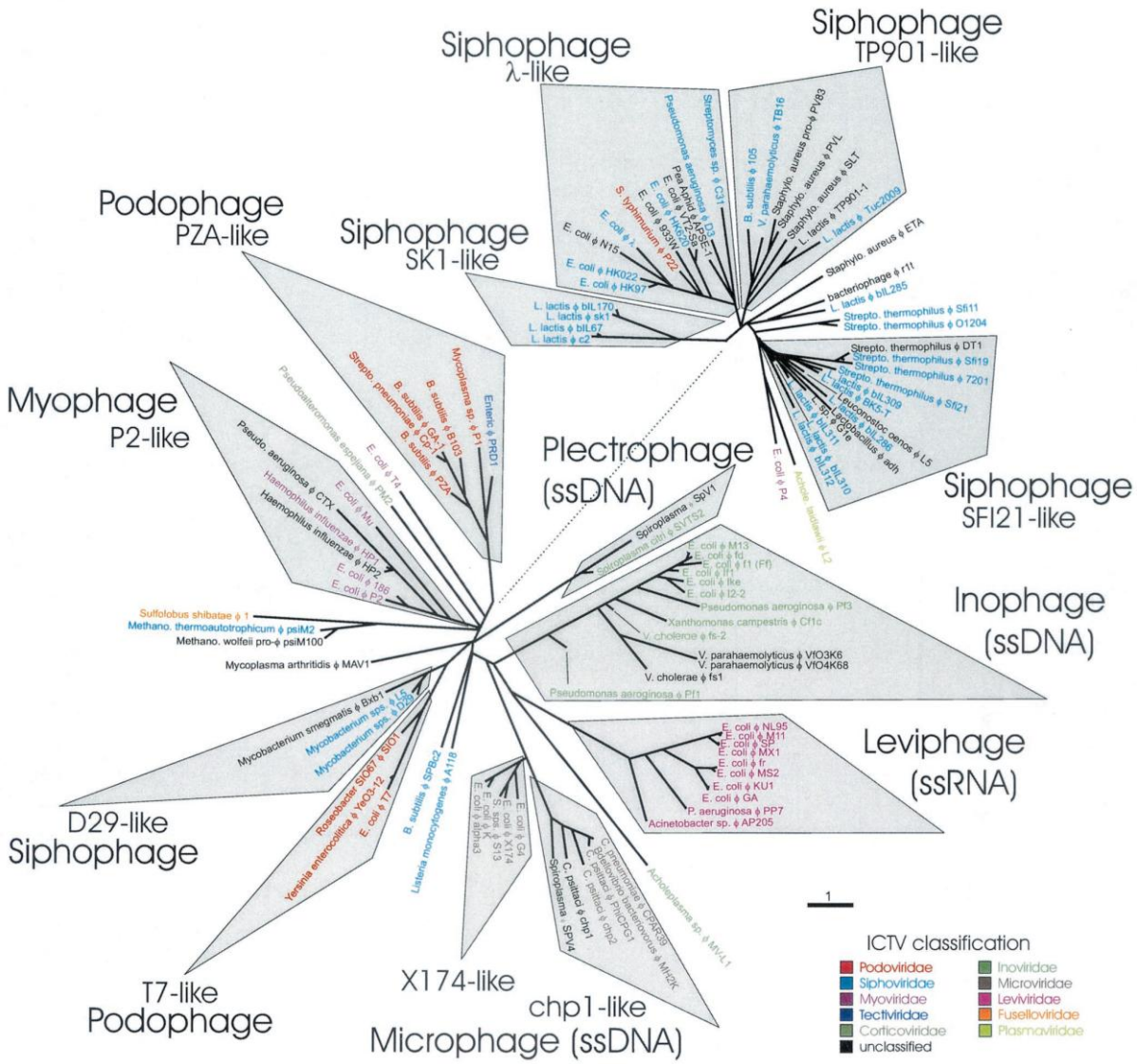

Figure 1.6 The Bacteriophage Proteomic Tree. Based on protein distance scores and coloured by International Committee on Taxonomy of Viruses (ICTV) classification. The dotted line indicates where the large Siphophage connects to other groups. Reproduced from Rohwer and Edwards (2002).

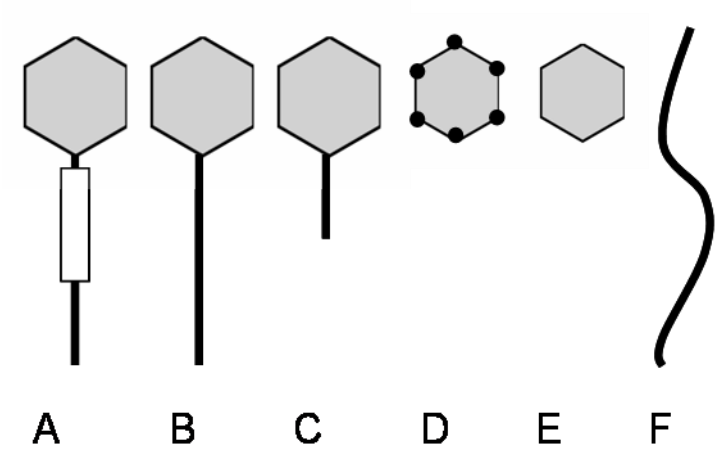

Figure 1.7 Basic morphological types of bacteriophage. All bacteriophage have a protein based capsule that houses the genome, which may be dsDNA (A, B, C), ssDNA (D & F) or ssRNA (E). Bacteriophage can range in size from 24-200 nm in length (Mayer, 2010). Summarised from Bradley (1967).

1.4.2 Bacteriophage Infection Cycle

Bacteriophage are historically classed as lytic or temperate (lysogenic), however, new terminology has been introduced to better define bacteriophage characteristics as either productive, reductive or destructive (Abedon, 2008) (Table 1.3).

Lytic bacteriophage kill their bacterial host cell by disruption of the cell membrane, whereas temperate bacteriophage produce a prophage (bacteriophage genome) that inserts and replicates within the host bacterial chromosome (Figure 1.8) (Todar, 2008).

Table 1.3 Glossary of terms to define bacteriophage characteristics.
Summarised from Abedon (2008).

Term	Definition
Lytic	Bacteriophage infections terminated following the production of bacteriophage virions by a bacteriophage-induced lysis of bacteria host.
Chronic	Release of produced virions without immediate destruction of host infection
Lysogenic	Bacteria remain viable, bacteriophage genome replicated but no virion produced.
Pseudolysogenic	No bacteriophage genome replication, but retains potential for future bacteriophage and virions production. Also known as carrier state.
Restrictive	Anti-bacteriophage immunity, involving the loss of bacteriophage viability but not bacteria.
Abortive	Loss of both bacteria and bacteriophage viability.

The lytic bacteriophage lifecycle can be summarised into four stages (Figure 1.8).

1. Infection is initiated upon adsorption of the bacteriophage to the bacterial cell, where the presence of adsorption cofactors determines the specificity of the bacteriophage. Bacteriophage adsorb to bacteria cells by the tips of their tail adhesions (MacGregor, 2008). It is often classified as a first-order reaction, where rate of adsorption is proportional to the concentration of bacteriophage and to the bacterial surface area. This first step is reversible, and temperature independent. It involves the formation of salt linkages between charged groups on the bacteriophage and bacteria cell surface.

This step is limited by the salt concentration in the growth medium, but also pH and temperature.

2. The bacteriophage then injects viral DNA into the bacteria host in the penetration phase.

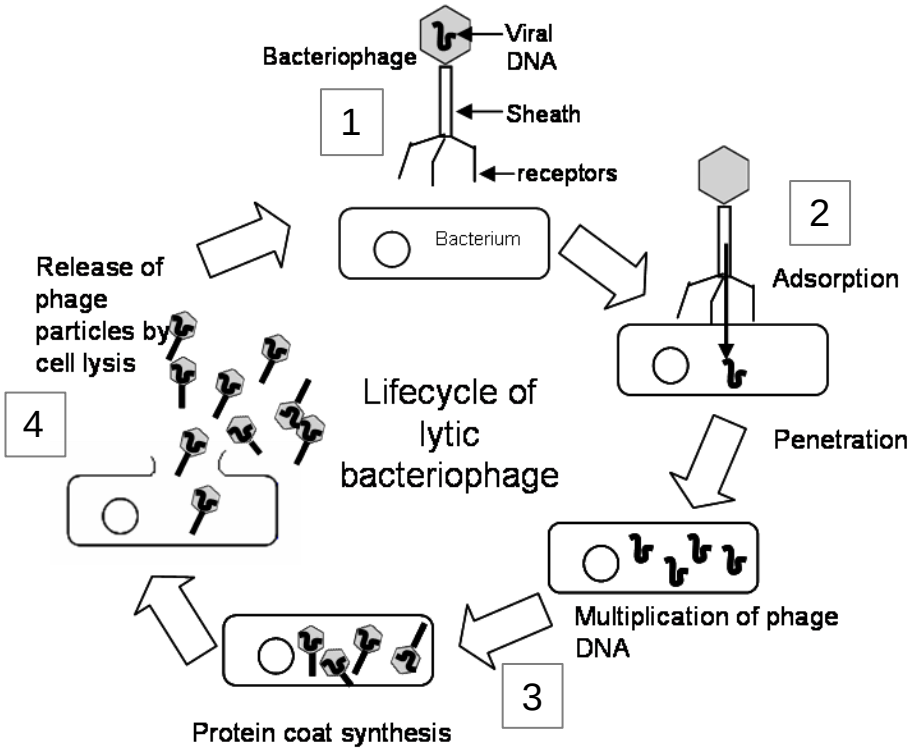

Figure 1.8 Diagram of the lifecycle of a lytic bacteriophage. Summarised from Todar *et al* (2008). Numbers refer to description of stages in Section 1.4.2.

3. The multiplication phase occurs in two stages which are linked to the expression of early and late bacteriophage proteins. The early proteins mediate bacterial cell wall repair, degradation of bacterial DNA and bacteriophage replication (Todar, 2008). During the late protein step, enzymes and proteins needed for completion of the bacteriophage structure and to assist in the escape from the bacterial cell are formed.

4. Once multiplication is complete, there are two mechanisms by which bacteriophage break open the bacterial cell wall to release their progeny into the environment. Large dsDNA bacteriophage utilise the holin-endolysin system (Bernhardt *et al.*, 2002). This is a protein (holin) and enzyme (endolysin) pair that degrade the bacterial cell wall, with timing controlled by the holin (Young *et al.*, 2000). Smaller ssDNA bacteriophage, however, have a single lysis gene. Once released from the bacteria, the mature bacteriophage can infect other bacterial cells. This complete lifecycle takes about 25-35 minutes to complete for a T4-bacteriophage (Todar, 2008). The virulence of a bacteriophage is affected by latent period and effective burst size. The first is the minimum time between adsorption of bacteriophage to host and lysis of the host cell with release of bacteriophage progeny. Burst size is the average number of bacteriophage released per infected bacterium (Abedon, 2008).

1.4.3 Bacteriophage Therapy

Bacteriophage are used with the objective of reducing the size of a bacterial population by the administration of specific bacteriophage to bacterial infections (Goodridge & Abedon, 2003). When treating bacterial infections in animals with bacteriophage, this is referred to as bacteriophage therapy, whereas when the target host is not an animal, the term biocontrol is more frequently used. Bacteriophage therapy is advantageous in response to the evolved resistance of bacteria to chemicals and antibiotics. Therapy usually exploits lytic bacteriophage as a single bacteriophage particle can infect and kill bacteria, in doing so self-replicating up to 200 times, which increases with each infection cycle. Alternatively, lysogenic bacteriophage have been exploited by companies such as Novolytics Ltd, as an alternative approach to treating bacterial infections (Novolytics Limited, 2011). Despite obvious potential, bacteriophage use is still controversial (Stone, 2002; Dabrowska *et al.*, 2005) due to lack of quantitative research and knowledge of the three 'dynamic components': infected human, infecting bacteria and bacteriophage (Merril *et al.*, 2003). Human application is only available in the European Union from

the Institute of Immunology and Experimental Therapy, Wroclaw (Hausler, 2006). Currently a number of Western companies are developing, licensing and selling bacteriophage-based products, such as BigDNA (bacteriophage DNA vaccinations), Biophage Pharma Inc. (environmental therapies and diagnostics) and Intralytix (food safety) (Housby & Mann, 2009), with some bacteriophage products receiving 'generally regard as safe' (GRAS) status (Hagens & Loessner, 2010).

1.4.3.1 Examples of Bacteriophage Therapy and Biocontrol

There are a number of commercially available bacteriophage products for the treatment of bacterial infections, especially within the food industry. Listex is a product produced by EBI Food Safety to target *Listeria monocytogenes* in food products. Livestock have been treated for a range of bacterial infections, including *Staphylococcus* infections causing mastitis in cattle (Gill *et al.*, 2006), septicaemia and meningitis caused by *Escherichia coli* in calves and chickens (Barrow *et al.*, 1998) and respiratory tract infections (Nelson *et al.*, 2001). Advanced Pest Solutions Ltd, this project's commercial partner, is developing a bacteriophage platform technology to specifically target agricultural bacterial pathogens, in addition to associated arthropod pests (A Blackwell, *pers. comm.*).

In vivo toxicity tests are required before use of bacteriophage therapy becomes available in many commercial settings. To date, however, no side affects in chickens (Oliveira *et al.*, 2006) or humans have been observed (Bruttin & Brussow, 2005).

1.4.3.2 Advantages and Limitations of Bacteriophage Use

Bacteriophage have a number of properties that make them a suitable candidate for the management of sheep scab disease (Table 1.4).

Table 1.4 Beneficial properties and limitations of using bacteriophage as a general therapeutic agent. References:(Smith & Huggins, 1982; Levin & Bull, 1996; Muniesa & Jofre, 1998; Buckling & Rainey 2002; Boeke, 2004; MacGregor, 2008; Scott & Mattey, 2009).

	Bacteriophage	Comments
Advantages	They are very specific, with narrow host ranges, as they bind to cell receptors. This means that they are not harmful to non-targeted bacteria, humans or animals.	Antibiotics have broad range effects, which risk damage to host beneficial bacteria.
	They are self replicating, unlike antibiotics (Levin & Bull 1996). A bacterium can be infected and lysed by a single bacteriophage particle.	A single application of bacteriophage may be sufficient
	They are effective against multi-drug resistant bacteria (Boeke 2004).	
	Bacteriophage will decline rapidly and naturally when bacteria are no longer present (Williams & Huggins 1982) terminating any further effects of the bacteriophage.	The ecosystem will not be harmed by an influx of bacteriophage
	There have been no reports of allergic reactions triggered by their use and they have few side effects.	
	They are cheap and easy to produce, usually by fermentation (MacGregor 2008).	
	They are 'environmentally friendly' as the bacteriophage species used will be isolated from the environment hence nothing novel or foreign is introduced into the ecosystem.	
	Bacteriophage can overcome bacterial resistance by mutating (Buckling & Rainey 2002).	Organophosphates and antibiotics are unable to do this.
	Methods are being developed to immobilise bacteriophage onto substrates, enabling long term storage of bacteriophage. Patent for immobilisation & stabilisation of virus (Scott & Mattey, 2009).	
Limitations	The method of bacteriophage delivery and spatial distribution to the infection is very important (Levin & Bull 1996). Bacteriophage cannot actively seek bacteria and a successful infection requires the bacteriophage to be in the vicinity of the bacteria.	
	Their specificity requires accurate identification of the target bacteria.	
	Some bacteriophage have beneficial associations with bacteria including the ability to integrate their DNA and produce toxins (MacGregor 2008).	One example of this is Shiga toxin produced by bacteriophage that increases the pathogenicity of *Escherichia coli* O157:H7 (Muniesa & Jofre 1998).

1.5 Project Aims

There is a genuine need for an alternative method of controlling *Psoroptes ovis* mites. There are weaknesses in the present controls, owing to the emergence of resistance and human health concerns. This thesis aims to investigate the bacterial associations of *P. ovis* mites and the potential of using bacteriophage to form a novel method of biocontrol for this disease.

The thesis is separated into three main experimental chapters, with the following objectives:

i. to identify the microbial environment associated with *P. ovis* mites and sheep scab-infected fleece;
ii. to investigate the relationship between *P. ovis* and identified bacteria on mite survival;
iii. to isolate bacteriophage from the environment infective towards *P. ovis*-associated bacteria and assess the affect on mite survival.

2 Microbial Environment of Sheep Scab

2.1 Introduction

2.1.1 Bacterial Associations of Arthropods

Psoroptes ovis mites spend their entire lifecycle on sheep. Living in such close association with the fleece, the microbial composition of this environment is a logical starting point for the identification of potential microbial symbionts for the control of scab mites. Moreover, bacteria present on sheep skin may infect wounds caused by *P. ovis* presence (Baker & York, 1999), increasing the pathogenicity of the disease (Mathieson & Lehane, 1996; Bates, 1999a; Hogg & Lehane, 2001). Therefore, in addition to targeting mites directly, the lesions may also be potential targets for novel antimicrobials to reduce the impact of the disease.

Endosymbionts, organisms that live within another organism, may have either mutualistic, commensal or parasitic effects on its host. These endosymbiotic organisms are known to play a central role in the survival, development, ecology and evolution of their arthropod hosts (Brune, 2003). Removal or reduction of these microbes can often result in abnormal development and reduced survival of the arthropod host (Eutick *et al.*, 1978a; Hogg & Lehane, 1999; Fukatsu & Hosokawa, 2002). Detailed examples of their roles are given in Chapter 1, Section 1.3.1. Briefly, these bacteria may be beneficial, providing better nutrition (Buchner, 1965), heat tolerance (Dunbar *et al.*, 2007), pathogen resistance (Hedges *et al.*, 2008; Zouache *et al.*, 2009a) and protection against non-indigenous gut species (Dillon & Dillon, 2004). Alternatively they may be pathogenic (Min & Benzer, 1997) and exhibit negative reproductive effects on the host (Zchori-Fein *et al.*, 2001; Zouache *et al.*, 2009b). Microbial symbionts may also be involved in communication or to convey signals between hosts, such as the use of *Klebsiella* by house flies (*Musca domestica)* (Lam *et al.*, 2007).

Endosymbiont effects can also be largely dependent on environmental factors, even switching between mutualist- and pathogen roles depending on environmental conditions, such as the secondary symbiont *Cardinium* in spiders (Duron *et al.*, 2008). Endosymbionts may be obligate (permanent) or transient and be transmitted vertically, from mother to offspring or horizontally, between hosts (Zchori-Fein & Perlman, 2004; Wernegreen, 2004). A characteristic of endosymbionts is their reduced genome sizes, an evolutionary artefact of their intracellular lifestyle, with the smallest known cellular

genome (144 kb) possessed by *Candidatus Hodgkinia cicadicola*, a symbiont of the cicada (*Diceroprocta semicincta)* (McCutcheon *et al.*, 2009).

Endosymbionts have been detected in a diverse range of arthropods, including mites, such as the predatory mite (*Metaseiulus occidentalis*) and poultry red mite (*Dermanyssus gallinae*) (Table 2.1). One such endosymbiont is *Cardinium,* a member of the Cytophaga-Flavobacterium-Bacteroidetes (CFB) phylum (Zchori-Fein *et al.*, 2001; Zchori-Fein & Perlman, 2004). This bacterium has been shown to have effects on reproduction as well as being a beneficial gut symbiont. It is restricted to three arthropod lineages, including the Acari, of which *P. ovis* is a member (Weeks & Stouthamer, 2004; Hoy & Jeyaprakash, 2008; Moro *et al.*, 2009; De Luna *et al.*, 2009). For this reason this bacterium may also be present in *P. ovis* mites.

In the search for a candidate for microbial control of scab mites, heritable endosymbionts would have the greatest potential. This is due to the highly conserved relationship between endosymbiont and host, in addition to a greater likelihood of the endosymbiont being present in all populations of *P. ovis* mites, irrespective of geographic location. Although present in arthropods, some endosymbionts are unsuitable targets for arthropod control as they are also found in mammals. One example is *Bacteroidetes,* which is found both in the predatory mite *M. occidentalis*, but also in the mammalian gut (Hoy & Jeyaprakash, 2005).

Although a number of bacterial species have been identified within *P. ovis* (Section 1.3.2.2), it is unknown whether the bacteria present during scab infections are naturally occurring on the fleece or whether they are introduced *via* mite faecal pellets (Mathieson & Lehane, 1996; Beard *et al.*, 1998). Endosymbiotic bacteria have been detected previously in faecal pellets from other arthropods and due to the universal-structure of terrestrial arthropod digestive systems (Brody *et al.*, 1972) it is possible that *P. ovis* also excrete endosymbionts in this way.

Table 2.1 Endosymbiotic bacteria associated with arthropods and organisms related to *P. ovis* mites. Role/effects on host are given, if known. CI: cytoplasmic incompatibility. Table continued on page 37.

Phylum	Bacteria species	Known host organisms	Role	Reference
Alpha-Proteobacteria	*Wolbachia*	Predatory mite (*Metaseiulus occidentalis)*, Mosquito (*Aedes albopictus*)	Reproductive effects (CI)	Hoy and Jeyaprakash 2005, Zouache *et al* 2009
Beta-proteobacteria	*Comamonas* sp	Varroa mite *(Varroa destructor), Mosquito (Ae. albopictus)*	Gut symbiont	Nisbet & Blackwell 2009, Zouache *et al* 2009
	Delftia	Mosquito (*Ae. albopictus)*	Gut symbiont	Zouache *et al* 2009
Cytophaga-Flavobacterium-Bacteroides (CFB)	*Cardinium*	Predatory mite (*M. occidentalis),* poultry red mite (*Dermanyssus gallinae)*, biting midge (*Culicoides spp.),* spider mite (*Tetranychus spp*.)	Reproductive effects, symbiont	Hoy and Jeyaprakash 2005, De Luna *et al* 2009, Moro *et al* 2009
	Bacteroidetes	Predatory mite (*M. occidentalis)*	Reproductive effects	Hoy and Jeyaprakash 2005
Enterobacteriaceae	S-endosymbiont *(Sodalis glossinidius)*	Tsetse flies (*Glossina glossinidia*)	Gut symbiont (thiamine provision)	Beard *et al* 1993, Synder *et al* 2010

Table 2.1 continued.

Phylum	Bacteria species	Known host organisms	Role	Reference
Gamma Proteobacteria	*Enterobacter*	Predatory mite (*M. occidentalis)*	Gut symbiont	Hoy and Jeyaprakash 2005
	Wigglesworthia	Tsetse flies (*G. glossinidia*)	Gut symbiont (Vitamin B provision)	Aksoy 1994, 1995, Baumann and Moran 1997
	Rickettsiella	Poultry red mite *(D. gallinae*), Varroa mite *(V. destructor*),	Potentially pathogenic	De Luna *et al* 2009, Moro *et al* 2009, Nisbet & Blackwell 2009
	Pasteurella multocida	Poultry red mite (*D. gallinae)*	Saprophyte/opportunistic pathogen	Moro *et al* 2009
Proteobacteria	*Schineria* sp	Poultry red mite (*D. gallinae)*	unknown	Moro *et al* 2009
Tenericutes	*Spiroplasma*	Poultry red mite (*D. gallinae*), Tabanid flies (*Tabaninae spp*.)	Reproductive effects (male mortality)	De Luna *et al* 2009, Moro *et al* 2009,

The diversity of *P. ovis* gut/faecal bacteria is also of interest as the composition may reveal the transmission route of mite-associated bacteria. Bacteria may be transient through the gut where they are able to proliferate and then excreted onto the skin and fleece where they are indiscriminately ingested by another mite. If endosymbiotic bacteria are present within *P. ovis* mites, they may be vertically transmitted, involving the deposition of bacterial substrates onto or into eggs (Lam *et al.*, 2007) or horizontally transmitted (Darby & Douglas, 2003), as *P. ovis* mites live in extremely close contact with each other. One method used in this chapter to investigate the contributions by *P. ovis* to skin bacteria are gut faecal trails. *P. ovis* mites were first surface sterilised to ensure bacteria are derived from internal mite cavities (Meyer & Neurand, 1991; Beard *et al.*, 1998; Graf & Ruby, 1998; Banjo *et al.*, 2005) and then excreted bacteria were identified.

Identification of the microbial composition of sheep fleece has been carried out previously and a shift in microbial diversity often coincides with incidences of disease (Merritt & Watts, 1978a; Merritt, 1980; Tadayon *et al.*, 1980; Chin & Watts, 1992; Lyness *et al.*, 1994) (Section 1.3.2.1). Variations associated with microbial communities have also been related to geographical diversity (Hogg & Lehane, 2001), as well as breed differences, which are in turn affected by the properties of the fleece (Losson *et al.*, 1999). There are a number of genera that appear to be consistently associated with the fleece environment that have also been previously detected in *P. ovis* or related arthropods, such as the chewing louse, *Bovicola ovis,* which also infest sheep (Table 2.2). This thesis is, however, the first documented study to investigate sheep scab infected fleece.

Novel control strategies are urgently required for *P. ovis* mites. A number have been investigated and described in Section 1.2.3. Potential methods for the control of arthropods based on their bacterial associations include the modification of internal microbiota of arthropods to form transgenic hosts, which removes the ability of the arthropod to spread disease (Beard *et al.*, 1998) and the targeting of beneficial bacteria directly, by 'symbiont based control' (Douglas, 2007).

Table 2.2 Summary of bacteria isolated from sheep, *P. ovis* mites and *Bovicola ovis* lice in previous studies. (Merritt & Watts, 1978b; Murray & Edwards, 1987; Chin & Watts, 1992; Lyness *et al.*, 1994; Mathieson & Lehane, 1996; Hogg & Lehane, 1999; Hamilton *et al.*, 2003).

Major bacterial group	No. of bacteria species isolated from			
	Healthy fleece	Unhealthy fleece	*P. ovis* mites	*D. ovis* Lice
Staphylococcus sp	5	3	2	1
Streptococci sp	0	3	1	
Micrococcus sp	1	2		1
Pseudom/Alcaligenes sp	3	10	1	1
Bacillus sp	3	2	1	
Acinetobacter/Moraxella sp	1	3		
Corynebacterium sp		4		
Enterobacter sp	2	6	2	1
Arcanobacter sp		1		
Flavimonas sp		1		
Actinomyces sp		1	1	

2.1.2 Microbial Community Analysis Techniques

A number of techniques have been used to investigate the microbial communities associated with a wide range of arthropods, including members of the Acari; predatory mites (*Metaseiulus occidentalis*) (Hoy & Jeyaprakash, 2005), house dust mites (*Dermatophagoides farina)* (Valerio *et al.*, 2005), orbatid mites (*Archegozetes longisetosus*) (Acari: Oribatida) (Seniczak *et al.*, 2009) and scab mites (*P. ovis*) (Mathieson & Lehane, 1996; Hogg & Lehane, 1999; Hogg & Lehane, 2001). Historically, electron microscopy (Kitajima *et al.*, 2007) and biochemical tests and phenotypical methods (morphology, Gram reaction, presence of enzymes and anaerobic growth) were used to classify bacteria present in a sample. Microbiological culturing has limitations as it only selects a small fraction of the organisms to grow (Nocker *et al.*, 2007), thereby underestimating microbial diversity, structure and interactions of unculturable bacteria. For example, Torsvik *et al.,* (1990) estimated that only 0.3% of soil bacteria are culturable (compared to total cell counts).

In addition, the effect of development stage on microbial communities has been investigated; such as nymphal stages of triatomines bugs (*Rhodnius prolixus*) (Azambuja *et al.*, 2004) and aquatic crane-fly (*Tipula abdominalis*) (Klug & Kotarski, 1980). Biochemical

tests have previously been used to analysise the composition of arthropod faecal bacteria (Mathieson & Lehane, 1996; Oravecz *et al.*, 2002).

Currently, molecular techniques are favoured for microbial identification, with most methods starting with amplification of a target gene by polymerase chain reaction (PCR) or direct cloning and sequencing (shotgun sequencing) (Nocker *et al.*, 2007). PCR is very sensitive, with the ability to amplify very low concentrations of DNA. Alvarez *et al.,* (1995) reported detection from an equivalent of one bacterial cell per PCR reaction. The products can then be separated by either sequence or length, by a number of different techniques, including restriction fragment length polymorphism (RFLP), (automated) ribosomal intergenic spacer analysis ((A)RISA) and denaturing gradient gel electrophoresis (DGGE). A comprehensive review of these techniques was produced by Nocker *et al.,* (2007). A summary of advantages and limitations of each is given (Table 2.3). The majority of these techniques use agarose or polyacrylamide gels to separate the products. This is a useful tool as many samples can be run simultaneously and bands can be excised directly from the gel for further sequencing or downstream analysis (Theron & Cloete, 2000). Polyacrylamide gels, although offering high resolution separation, can be laborious and difficult to produce in the laboratory (Nocker *et al.*, 2007).

The predominant target for molecular fingerprinting of bacterial communities is the 16S rRNA gene (Kohne, 1986; Ludwig & Schleifer, 1994; Kolbert & Persing, 1999). The 16S rRNA gene in addition to 23S and 5S rRNA genes, make up the *rrn* operon which has an internal transcribed spacer (ITS) region between the 16S and 23S genes. These gene targets provide both conserved regions (in the form of the 16S gene) and variable regions (length of the ITS region). Primers can be designed for the conserved regions to amplify DNA and then the products can be separated by sequence heterogeneity to provide phylogenetic differentiation (Garcia-Martinez *et al.*, 1999) and bacterial identification (Kolbert & Persing, 1999; Cardinale *et al.*, 2004). RISA compares microbial communities based on the amplification of different ITS regions (Garcia-Martinez *et al.*, 1999; Fierer *et al.*, 2005).

To detect the presence of specific bacteria, such as endosymbionts, the 16S rRNA gene can also be used. For some endosymbiont bacteria, alternative genes may be used, such the *wsp* gene (*Wolbachia* surface protein) for *Wolbachia*, which provides better differentiation (Braig *et al.*, 1998).

For optimisation and running of PCR assays, a positive control is required for accuracy. In this chapter, DNA extracted from *Delia radicum* (cabbage root fly) and *Delia coarctata* (wheat bulb fly) were used as potentially positive endosymbiont controls. The presence of two endosymbionts, *Wolbachia* and *Cardinium*, have previously been detected in these organisms (Cordaux *et al.*, 2008).

Table 2.3 Summary of molecular techniques used for microbial community analysis. Method of separation of PCR products is given and advantages and limitations associated with each technique. Continued on page 42. References:(Fischer & Lerman, 1983; Suzuki & Giovannoni, 1996; Fisher & Triplett, 1999; Theron & Cloete, 2000; Crosby & Criddle, 2003; Fierer & Jackson, 2006; Nocker *et al.*, 2007).Continued on page 43.

Method	Abbr.	Name	Advantages	Limitations	Reference
Length	RFLP (a.k.a ARDRA)	Restriction Fragment Length Polymorphism (Amplified ribosomal DNA restriction analysis)	Robust index of bacterial diversity, generally consistent with results from clone libraries. Commonly used to cluster, isolated bacterial species into genotypic groups.	Complex profiles are produced, which can be difficult to analyse. Limited sensitivity of staining with some DNA dyes. Choice of enzyme is important. Cannot be used to measure diversity.	Nocker *et al* 2006.
	T-RFLP	Terminal-Restriction Fragment Length Polymorphism	Better suited for analysing a large number of samples and for quantitatively detecting differences in the diversity and composition of highly complex soil bacterial communities. Fluorescence simplifies sizing of fragments as only single band produced. Each band equals one species. Can use more than one restriction enzyme.	Underestimates total bacterial diversity as the method resolves only a limited number of bands per gel (<100) and bacterial species can share phylotypes. Must be careful if more than one enzyme is used as can overestimate species if not digested properly. High running costs.	Fierer & Jackson 2006
	RISA	Ribosomal Intergenic Spacer Analysis	Relatively low running costs and technique is straightforward. Can increase resolution at a strain level. Separates species by length of 16-23S ITS. Highly reproducible and can sequence bands directly from gels.	Possibility of overestimation caused by multiple copies of the ribosomal operon found within many bacterial genomes.	Fisher & Triplett 1999, Suzuki & Giovannoni 1996, Crosby & Criddle 2003
	ARISA	Automated-Ribosomal Intergenic Spacer Analysis	Adds fluorescence for automated detection of RISA fragments. Saves time as gels are unnecessary and can carry out computer-based analysis.	Potential for preferential amplification of shorter templates	Fisher & Triplett, 1999

Table 2.3 Continued.

Method	Abbr.	Name	Advantages	Limitations	Reference
Sequence	DGGE	Denaturing gradient gel electrophoresis	Denature DNA then separates by size on a gel.	Primer design is difficult as maximum length is much shorter than other methods (500bp). More than one species may migrate at same speed. Technique is laborious. Can be problems of significant background staining affecting analysis.	Fischer & Lerman 1983, Nocker *et al* 2006.
	TGGE	Temperature gradient gel electrophoresis	Denature DNA and then separate using melting behaviour. Can highly resolve differences.	As DGGE.	
Gel based			Can directly cut out bands for sequencing/analysis. Many samples can be run simultaneously in a single run.	Some gel types (e.g. polyacrylamide) can be laborious and difficult to produce.	Theron & Cloete 2000, Nocker *et al* 2006.

2.1.3 Summary and Aims

The microbial environments of *P. ovis* and sheep scab disease are investigated in this chapter using a number of methods:

- biochemical and phenotypical tests, in addition to PCR and cloning techniques, are used to compare the culturable bacteria present in healthy- and scab-infected fleece;
- the microbial communities associated with *P. ovis* mites are investigated using faecal gut trails, PCR assays, RISA and cloning techniques;
- endosymbiont-specific PCR assays are also used to investigate the presence of potential arthropod symbionts within *P. ovis*.

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Chemicals and Microbiological Media

All chemicals used were of molecular grade and sourced from Sigma (Sigma Chemical Co., UK) unless stated otherwise. Selective microbiological media; Nutrient Agar (NA; CM0003), Lamb serum agar ((LSA; NA supplemented with 10% lamb serum (Invitrogen)), Pseudomonas agar (PA; CM559), MacConkeys agar (MAC; CM0007) and de Man, Rogosa, Sharpe agar (MRS; CM0325) were sourced from Oxoid, UK and made according to manufacturer's instructions.

2.2.1.2 Fleece Samples

Infected Fleece Samples

Fleece samples from natural sheep scab infections were received from one of eight Scottish Agricultural College (SAC) Disease Surveillance Centres throughout Scotland, following positive diagnosis for sheep scab. Samples were received between February 2008 and January 2009 (Table 2.4) and were mostly Blackface or crossbreed sheep, although some were not known. On receipt, samples were kept in plastic falcon tubes at 4°C until use (Lyness *et al.*, 1994).

Healthy Fleece Samples

All healthy fleece samples were collected from Suffolk and Blackface pregnant ewes at the SAC Easter Bush Estate, Edinburgh (February 2008). The sheep were part of a separate nutritional study and had no prior history of, or current sheep scab infection. On receipt, samples were kept in plastic falcon tubes at 4°C until use (Table 2.4).

2.2.1.3 *P. ovis* Mite Samples

From Natural Sheep Scab Infections

Mite samples (mixture of males and females) were received with infected fleece samples from SAC Disease Surveillance Centres throughout Scotland (Section 2.2.1.2). On receipt, mites were frozen at -80°C for subsequent analysis (Table 2.4).

From *in vivo* cultured Sheep Scab Infections

Mite samples (mixture of males and females) were received from a long-term *in vivo* culture maintained at The Moredun Research Institute, Edinburgh. Samples were received 24- to -48 h following harvest from sheep. Mites were either used immediately for faecal-trail experiments (Section 2.2.2.5), *in vitro* feeding experiments (Chapter 3, Section 3.2.2.5), or frozen at -80°C for molecular analysis (Section 2.2.2.7).

2.2.1.4 *Delia* spp. Fly samples for PCR controls

Wheat bulb fly (*Delia coarctata*) and cabbage root fly (*Delia radicum*) collected from Midlothian, Scotland, were supplied by Mr C Rogers and Mr W Deasy respectively (Scottish Agricultural College, Edinburgh).

Table 2.4 Origin and date of receipt of fleece and *P. ovis* samples used for bacterial identification.

					Samples used for:				
	Origin	Sample	Location	Date collected	Fleece biochemical tests	*P. ovis* faecal trails	Bacteria Group-Specific PCR	ITS-specific PCR	Endosymbiotic-specific PCR
Fleece	Natural Scab infection	S1	Dumfries	Feb-08	x				
		S2	Thurso	Feb-08	x				
		S9	Thurso	Aug-08				x	
		S14	Thurso	Sep-08				x	
		S22	Thurso	Dec-08					
		S23	Dumfries	Jan-09				x	
	Healthy fleece	F1	Edinburgh	Feb-08	x				
		F24	Edinburgh	Feb-08	x			x	
		F38	Edinburgh	Feb-08	x				
		F41	Edinburgh	Feb-08	x				
		F60	Edinburgh	Feb-08	x				
		F70	Edinburgh	Feb-08	x				
		F88	Edinburgh	Feb-08	x				
		F91	Edinburgh	Feb-08	x			x	
		F109	Edinburgh	Feb-08	x			x	
		FA	Aberdeen	Feb-08	x				
Mites	*In vivo* culture	M1	Edinburgh	May-08		x	x	x	x
		M2	Edinburgh	Aug-08			x	x	x
		M3	Edinburgh	Oct-08			x	x	x
		M4	Edinburgh	Jul-09					x
		M5	Edinburgh	Sep-09					
		M6	Edinburgh	Oct-09					
		M7	Edinburgh	Nov-09					
		M8	Edinburgh	Aug-10					
	Natural infection	S1	Dumfries	Feb-08			x		x
		S4	Dumfries	Jun-08			x		
		S7	Perth	Jul-08			x		
		S11	Dumfries	Sep-08					x
		S19	Edinburgh	Dec-08				x	x
		S21	Thurso	Dec-08				x	x
		S22	Thurso	Dec-08				x	
		S29	Dumfries	Jan-09					x

2.2.2 Methods

2.2.2.1 Bacterial Isolation from Fleece- Fleece Washing

Bacteria were isolated from sheep fleece following the method of Lyness *et al.,* (1994). Briefly, fleece (approximately 0.6 g) was added to 10 ml of Ringers' solution (supplemented with 0.2% peptone) in a 15 ml falcon tube containing 10 x 3 mm sterile glass beads. The sample was vortexed for 1 min and then agitated on an orbital shaker for 10 min at room temperature (RT). The fleece-wash supernatant was used for bacterial identification (Section 2.2.2.4).

2.2.2.2 Bacterial Growth on Selective Media

To maximise diversity of bacteria cultured, sheep fleece-wash supernatant (Section 2.2.2.1) was ten-fold serially diluted in Ringers' solution (with 0.2% peptone) then 100 µl was plated onto NA (a general purpose medium), LSA, PA (suitable for *Pseudomonas* species), MAC (suitable for coliforms and enteric bacteria) and MRS (favours lactobacilli), in duplicate (Section 2.2.1.1). Plates were aerobically incubated overnight at 27°C. MRS agar plates were incubated for an additional 24 h to allow for slower growing lactobacilli organisms (Bridson, 1990). Unique colonies were picked and purified by two rounds of streaking onto the same agar-type used for isolation.

2.2.2.3 Glycerol freezer stocks – Fleece Bacteria Samples

Glycerol stocks were made for each bacterial isolate (Section 2.2.2.2) using 800 µl of overnight bacterial culture and 200 µl sterile glycerol. Stocks were then stored at -80°C for future analysis (Section 2.2.2.7).

2.2.2.4 Bacterial identification

Purified bacterial colonies were identified to a group level following standard microbiological and biochemical tests (Appendix 1).

2.2.2.5 Bacterial Isolation from *P. ovis* Faecal Trails

P. ovis faecal trail experiments were carried out following the method of Mathieson (1995). Briefly, mites were surface sterilised by submersing in 100% acetone for 30 s followed by 70% methanol for 60 s. Sterilised mites were tested for external contamination by streaking across NA plates and incubated under the same conditions as the samples. Individual mites were placed in the centre of different selective media plates (NA, LSA, PA, MAC, MRS) using a sterile mounting needle, in duplicate for each

plate type. Plates were loosely sealed with Parafilm M (Alpha Laboratories, UK) and incubated at 20°C for 2 d. Unique colonies were picked and purified by two rounds of streaking onto the same agar-type used for isolation. Bacteria were identified using biochemical tests as previously described (Section 2.2.2.4) and by cloning and sequencing of the ITS region (Section 2.2.2.7). From here these bacterial isolates will be referred to as mite faecal bacteria (MFB).

Mite Faecal Bacteria Freezer Stocks

A single bacterial colony isolated from mite faecal trails (Section 2.2.2.5) was used to make cryobead stocks (PROTECT bacterial preservers, Technical Service Consultants Ltd., UK) following manufacturers instructions and stored at -80°C for future analysis (Section 2.2.2.7).

Maintenance of Mite Faecal Bacteria.

Stocks of MFB for experimentation were maintained on nutrient agar plates (NA). Isolates were re-streaked fortnightly onto new NA plates, incubated at 27°C overnight then stored at 4°C. After three sub-cultures from stock plates, bacteria were re-plated from -80°C cryobead stocks (Section 2.2.2.5). To prepare liquid cultures of MFB, single colonies were picked from NA stock plates with a sterile loop and inoculated into 30 ml nutrient broth (NB). Cultures were incubated overnight at 27°C, on an orbital shaker. Cultures were then stored at 4°C until use for a maximum of one week.

2.2.2.6 DNA Extraction for Polymerase Chain Reaction (PCR)

Microlysis of *P. ovis*

DNA was extracted from a single mite using microlysis solution (Microzone Ltd, UK), which lyses cells to release DNA. For the microlysis treatment, 20 µl of microlysis solution was added to a single surface sterilised mite (Section 2.2.2.5). The sample was then heated for one cycle of 15 min at 65°C, 2 min at 96°C and 4 min at 65°C, followed by 1 min at 96°C, 1 min at 65°C and 30 min at 96°C in a Thermal cycler (BioRad). The product was then used for specific-group PCR (Section 2.2.2.7).

Phenol/Chloroform Extraction

This method was used to extract DNA from *P. ovis* mites, *D. radicum* and *D. coarctata* and fleece for ITS and endosymbiont-bacteria PCR. Whole *P. ovis* mites, *D. radicum* and *D.*

coarctata were initially surface sterilised and tested for residual surface contamination as previously described (Section 2.2.2.5).

For extraction ten mites, one fly (*D. radicum* or *D. coarctata*) or approximately 20 mg of fleece were transferred to sterile tubes containing sterile Ballotini beads (Thistle Scientific) and 440 µl of 2 X TENs extraction buffer added (pH 8.0; 0.8 mM Tris-base, 0.5 mM NaCl, 0.3 mM EDTA, 1 mg/ml phenanthroline, 1µl/ml mercaptoethanol, 0.02g/ml PVP). Samples were homogenised/agitated using a Fastprep machine (Thermo Electron Corporation) for 40 s at 6 ms^{-1} then placed on ice for 2 min and the process repeated three times. DNA extraction method subsequently followed a SAC standard operating procedure for phenol/chloroform DNA extraction (SAC SOP Lab/Mol/020). Briefly, to the homogenate, 400 µl SDS (2%) were added, vortexed briefly and incubated at 65°C for 30 min, following which 800 µl of phenol/chloroform/IAA (25:24:1) were added and centrifuged for 10 min (9447 x *g*). The aqueous supernatant was transferred to a mixture of 40 µl of 0.5 M ammonium acetate, 600 µl of isopropanol and 4 µl of GlycoBlue (Ambion) in fresh microfuge tubes and stored overnight at -20°C. Following overnight incubation, the tubes were thawed and centrifuged (9447 x *g*) for 20 min and the supernatant discarded. The pellet was washed with 600 µl of 70% ethanol, re-centrifuged (9447 x *g*) for 10 min and the supernatant discarded. Finally, the tubes were air dried in a laminar flow cabinet for 10 min and the pellet was then re-suspended in 50 µl of sterile distilled water, after which the tubes were placed in a 50°C water bath for 10 min to ensure DNA was completely resuspended. DNA quantity and purity was measured using a ND-1000 spectrophotometer (Nanodrop).

2.2.2.7 PCR

Presence of Bacterial DNA

Extracted DNA samples (from mites Section 2.2.2.6) were initially checked with universal bacteria primers, Eub338F and Eub528R (Fierer *et al.*, 2005) (Table 2.5) to assess the presence of bacterial DNA. Universal bacteria PCR was performed in a 25 µl reaction with 0.3 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1U GoTaq polymerase (Promega) with 2 µl of DNA. Cycling conditions were as Fierer *et al* (2005). For analysis, 8 µl of PCR product was run on a 2% agarose gel containing GelRed (Biotium) in TBE buffer (Eurogentec) with 100bp or $1Kb^+$ ladder for size calibration

(Invitrogen). Gels were run at 100 V and visualised using a ChemiImager (Alpha Innotech Corp).

Bacterial Group-Specific Primers

DNA extracted from mites (Section 2.2.2.6) was amplified with bacteria-specific primers (Table 2.5). PCR reaction mixtures and cycling conditions for each group-specific primer set were first optimised using a range of laboratory strain bacterial cultures received from K Stanley, APS Ltd. Cycling and reaction details are given in Appendix 2). All PCR assays included a positive bacteria control and two negative controls, water and a non-specific bacterium to check the specificity of the PCR and were run in duplicate. PCR products were visualised on an agarose gel as described in Section above (2.2.2.9).

Table 2.5 PCR primer sequences and amplicon length (bp) for bacterial group specific-PCR assays for *P. ovis* mites. Primers targeted 16S rRNA sequences.

Group	Examples	Gram	Name	Sequence 5'-3'	Reference	Approx Amplicon Length (bp)
General Bacterial		Both	Eub338F Eub518R	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	Fierer *et al* 2005	200
Firmicutes	*Bacillus,* *Lactobacillus,* *Staphylococcus* *Streptococcus*	+ + + +	Lgc353F Eub518R	GCA GTA GGG AAT CTT CCG ATT ACC GCG GCT GCT GG	Fierer *et al* 2005	180
Actinobacteria	*Actinomyces* *Micrococcus* *Nocardia* *Streptomyces*	+ + + +	S-C-Act-235-a-S-20 S-C-Act-878-a-A-19 Eub518R	CGC GGC CTA TCA GCT TGT TG CCG TAC TCC CCA GGC GGG G ALTERNATIVE REVERSE PRIMER, ATT ACC GCG GCT GCT GG	Stach *et al* 2003	640
α-Proteobacteria	*Acetobacter aceti*	-	Eub338F Alf685R	ACT CCT ACG GGA GGC AGC AG TCT ACG RAT TTC ACC YCT AC	Fierer *et al* 2005	365
γ-proteobacteria	*Pseudomonas*	-	Ps-for Ps-rev	GGT CTG AGA GGA TGA TCA GT TTA GCT CCA CCT CGC GGC	Widmer *et al* 1998	1000

ITS PCR

Microbial community analysis was carried out on *P. ovis* mites and fleece (healthy and scab-infected). Extracted DNA from fleece and mites (Section 2.2.2.6) was amplified using forward primer ITSF (5'-GTC GTA ACA AGG TAG CCG TA -3') and reverse primer ITSReub (5'-GCC AAG GCA TCC ACC-3') (Cardinale *et al.*, 2004) that targeted the bacterial 16S-23S internal transcribed spacer (ITS).

PCR was performed in a 25 µl reaction using 10 µl sterile water, 12.5 µl master mix (Promega; 1.5 mM $MgCl_2$, 200 µM dNTPs, 1U colourless Go Taq), 0.5 µM primers and 2 µl DNA using a GeneAmp Thermal cycler (Biometra). Cycling conditions consisted of 2.5 min at 94ºC, 30 cycles of 45 s at 94ºC, 1 min at 55ºC, 1 min at 72ºC, followed by a final extension of 7 min at 72ºC. 8 µl of PCR product was run on a 2% agarose gel containing GelRed (Biotium) in TBE buffer (Eurogentec) with 100bp or $1Kb^+$ ladder for size calibration (Invitrogen). Gels were run at 100 V and visualised using a ChemiImager (Alpha Innotech Corp). The remaining PCR product was either used for RISA (Section 2.2.2.10) or cloning and sequencing (Section 2.2.2.11).

Endosymbiotic Bacteria-Specific Primers

Primer sets specific for four endosymbiont bacteria were used to screen *P. ovis* mites (Table 2.6). All PCR reactions were conducted using Hotstart GoTaq (Promega, UK) and optimised PCR conditions are detailed in (Appendix 3). Cycling parameters for each primer pair were as detailed in the original primer reference. All PCR assays were run in duplicate and included *D. radicum* and *D. coarctata* extracted DNA as positive controls as endosymbiotic bacteria has been detected in them (Cordaux *et al.*, 2008), except *Wolbachia*-PCR which used positive *Wolbachia* DNA and a negative water control. After PCR amplification, 8 µl of PCR products were run on 2% agarose gels, with GelRed and visualised under UV light using a ChemiImager machine as before. To ensure validity of endosymbiont PCR amplification, positive bands of the expected band length were identified by sequencing (Section 2.2.2.11).

2.2.2.8 Ribosomal Intergenic Spacer Analysis

For Ribosomal Intergenic Spacer Analysis (RISA), the PCR products from ITS PCR (mites, healthy and scab-infected fleece; Section 2.2.2.7) were loaded into a DNA 1000 or DNA 7500 'On-chip-electrophoresis' following manufacturer's instructions (Agilent

Technologies). Briefly, the DNA sample is loaded onto a chip, in addition to internal markers and a fluorescent dye. The DNA amplicons are separated electrophoretically as on a gel yet fluorescence is then detected by a laser. An electropherogram, summarising the peaks of fluorescence and an electronic gel image, which both allow for accurate amplicon sizing, is produced. Dendrograms can then be produced to summarise the similarity of sample compositions.

Table 2.6 Primer sequences used for endosymbiont PCR screening assays with estimated amplicon length (bp). Cycling profiles were as original references. All primers targeted 16S rRNA sequences except *Wolbachia* (*Wolbachia* surface protein) (Braig *et al.*, 1998).

Endosymbiont	Primer name	Primer sequence	Expected band length	Primer reference
Cardinium	Ch-F	5' TAC TGT AAF AAT AAG CAC CGG C 3'	500 bp	Zchori-Fein *et al* 2010
	Ch-R	5' GTG GAT CAC TTA ACG CTT TCG 3'		
Comamomas	Com199F	5' CCT TGT GCT ACT AGA AGC 3'	433 bp	Zouache *et al* 2009
	Com614R	5' GCA GTC ACA ATG GCA GTT 3'		
Wolbachia	81F	5' TGG TCC AAT AAG TGA TGA AGA AAC 3'	500 bp	Braig *et al* 1998
	691R	5' AAA AAT TAA ACG CTA CTC CA 3'		
Rickettsia	EHR16SD	GGT ACC YAC AGA AGA AAG TCC 3'	345 bp	Brown *et al* 2001
	EHR16SR	TAG CAC TCA TCG TTT ACA GC 3'		

2.2.2.9 Cloning of PCR products

PCR product Purification

PCR products from ITS and endosymbiont-specific assays (Section 2.2.2.7) were purified using Roche High Pure PCR product (Roche) as per manufacturer's instructions. Quality and quantity were checked with the ND-1000 spectrophotometer and if at least 20 ng/µl of DNA with purity between 1.5- 2.0 was present, the samples were used for ligation.

Ligation

Purified PCR products were ligated into a pGEM-T Easy vector (Promega, UK) following manufacturer's instructions. Ligations were then either stored at -20°C or used for transformations.

Transformation and Selection of Positive Inserts

For the transformations, 3 µl of prepared ligations (Section 2.2.2.11) were mixed with 50 µl of JM109 High efficiency competent cells (Promega, UK). The mixture was incubated on ice for 30 min, followed by heat shocking cells in a 42°C water bath for 45 s and then immediately returned to ice for 5 min. To this, 450 µl of SOC medium (Invitrogen) were added then incubated with shaking at 37°C for 1 h 20 min

Transformed cells (100 µl) were then plated on LB- ampicillin plates (100 µg/ml), which had been previously spread with 100 µl of 0.1 M IPTG and 22 µl of 40 mg/ml X-Gal. Plates were incubated at 37°C for 12-18 h. Transformants were screened by blue/white selection and reference grid plates of positively transformed bacteria (white colonies) set up.

M13 PCR

To ensure positive transformation, the white colonies were screened with M13 primers, which amplify the pGEM-T vector region. Primers were M13 forward (5'- GTA AAA CGA CGG CCA G - 3') and M13 reverse (5'- CAG GAA ACA GCT ATG AC - 3') (Invitrogen). PCR was performed in a 25 µl reaction using 10 µl sterile water, 12.5 µl master mix (Promega; 1.5 mM $MgCl_2$, 200 µM dNTPs, 1U colourless Go Taq), 0.5 µM primers and 2 µl DNA. A cycling profile of 5 min at 94ºC, 5 min at 55 C, 35 cycles of 72ºC for 1.5 min, 94ºC for 45 s, 55ºC for 50 s, final extension of 72ºC for 10 min was

used. PCR products were checked on a 2% agarose containing GelRed (Biotium) in TBE buffer with 100bp or $1Kb^+$ ladder (Invitrogen) for size calibration. Gels were run at 100 V and visualised using a ChemiImager.

Plasmid Preparations

If inserts were detected following M13 PCR, a single bacterial colony was inoculated into 5 ml LB broth with ampicillin (100 μg/ml) and incubated overnight at 37°C with shaking. The bacterial cells were then pelleted and DNA extracted with either Wizard Plus SV Minipreps DNA purification system kit (Promega) or Hurricane mini prep (Gerard Biotech, OH) as per manufacturer's instructions. Purified plasmid DNA was then measured with a ND-1000 spectrophotometer. The DNA was also amplified with M13 primers as previously described and if bands were observed on agarose gel, samples were chosen for sequencing.

Sequencing and Analysis

DNA from plasmid preparations were sent to DBS Genomics, Durham University for sequencing (Applied Biosystems 3730 DNA Analyser). Received chromatograms were checked using Sequence Scanner v1.0 (Applied Biosystems) and primer/vector sequences were removed. Sequences were compared to published bacterial sequences using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990) and values of 50% query similarity or higher were used for identification.

2.2.2.10 Statistics

To compare relative bacterial abundance of fleece, CFU (per g of fleece) values were $\log_{10}$ (+1) transformed after Anderson-Darling normality test in Minitab v15 (MiniTab Inc.).

Shannon-weaver diversity assumptions could not be met to analyse bacterial diversity within fleece samples, therefore alternative analysis was carried out. To investigate the abundance and presence of species, the Peterson's homogeneity index (PHI) (Bakus, 1990; Garten, 2000) was calculated (Equation 2.1). For PHI, the number of individuals of each species was converted into a proportion (X_i) of the sample, then the mean homogeneity of the community was calculated in a pair by pair comparison, where

values close to zero indicated heterogeneous populations (Garten, 2000). Both were calculated using Excel 2007.

To analyse microbial communities of *P. ovis* and fleece, RISA profiles were used to generate matrices of band lengths in TotalLab (Agilent Technologies) and fleece samples were compared by Students' *t*-test (unpaired) and *P. ovis* samples compared by Mann-Whitney U test in MiniTab v15.

Equation 2.1. Peterson's homogeneity index (PHI). This accounts for both presence/absence and abundance of species between samples (a and b); i: individuals.

2.3 Results

2.3.1 Bacterial Isolation from Fleece

2.3.1.1 Relative Bacterial Abundance in Healthy and Scab-Infected Fleece

Fleece-washings were plated and individual bacterial colonies were counted, to calculate colony forming units (CFU) on five different agar types, in duplicate. Ten healthy samples were used (F1, F24, F38, F41, F60, F70, F88, F91, F109, FA) and two scab-infected samples (S1, S2). There appeared to be a higher density of bacteria from healthy fleece (median Log5.02 CFU/g) compared to infected fleece (median Log3.69 CFU/g) on all agar types, but due to small scab-infected sample size (n=2) this could not be determined statistically (Figure 2.1). There was little variation between the agar types, for healthy and scab-infected fleece, however, only healthy fleece samples produced bacterial colonies on MRS agar.

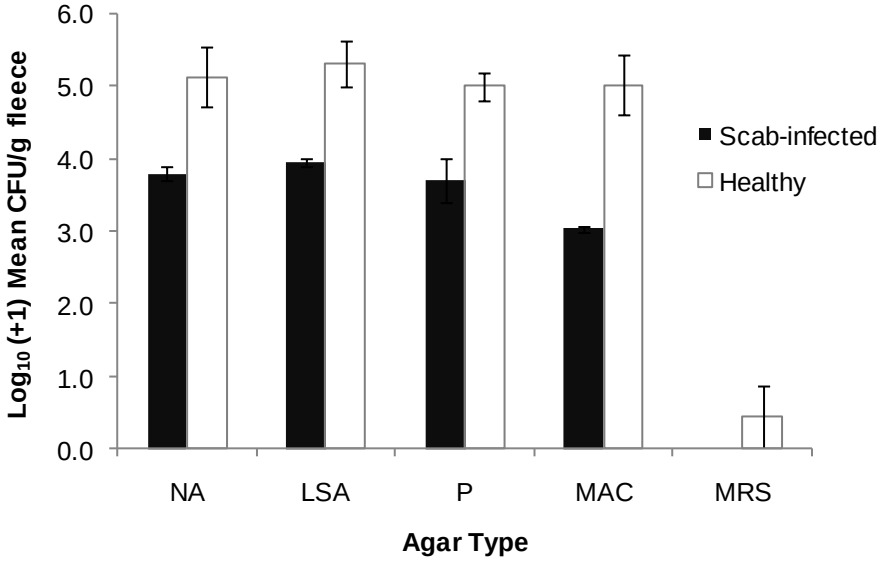

Figure 2.1 Log_{10} (+1) mean CFU/g of fleece for healthy (n=10) and scab-infected fleece (n=2). Five agar types: Nutrient agar (NA), Lamb serum agar (LSA), Pseudomonas agar (P), MacConkeys agar (MAC) and MRS agar (MRS). Error bars are standard error (s.e).

2.3.1.2 Bacterial Community Composition of Healthy and Scab-Infected Fleece

A wide diversity of bacterial colonies was isolated from healthy and scab-infected fleece samples used in Section 2.3.1.1. On average, 11 colonies were purified from each fleece

sample to give a total of 112 and 23 colonies from healthy and scab-infected fleece respectively. Each isolate was identified using phenotypical and biochemical characteristics to a major bacterial group (Appendix 4), as suggested by Cowan *et al.,* (2003) (Appendix Table A1.1). Nine colonies were unable to be classified following microbiological tests and identification table so were recorded as 'unidentified'; there were two Gram positive and seven Gram negative from five different samples (Appendix 4). Bacterial isolates from healthy fleece were classified as *Staphylococcus* (n=54), *Pseudomonas* (5), *Kurthia* (7), *Acinetobacter* (8), *Bacillus* (6), *Micrococcus* (16), *Streptococci* (8) and *Neisseria* spp. (8). Scab-infected bacteria only had representatives from *Staphylococcus* (n=4), *Pseudomonas* (11), *Bacillus* (1), *Micrococcus* (2) and *Streptococci* (5) (Figure 2.2).

Overall there were five major groups isolated from both healthy and scab-infected samples (*Staphylococcus, Streptococcus, Micrococcus, Bacillus, Pseudomonas*) and there were between three to seven different major bacterial groups isolated from a single fleece sample (Figure 2.2). To analyse the bacterial composition of fleece, the abundance of colonies of each bacterial group was analysed using the Peterson's homogeneity index (PHI). This calculates the proportion of each bacterial group of the whole community, where 0 indicates homogenous populations and 1.00 indicates heterogeneous populations. The two fleece states had a PHI of 1.00, indicating a heterogeneity between the two fleece types (Appendix 5). This method could only isolate culturable bacteria, so culture independent methods were also used to identify bacteria from fleece samples (Section 2.3.3.2).

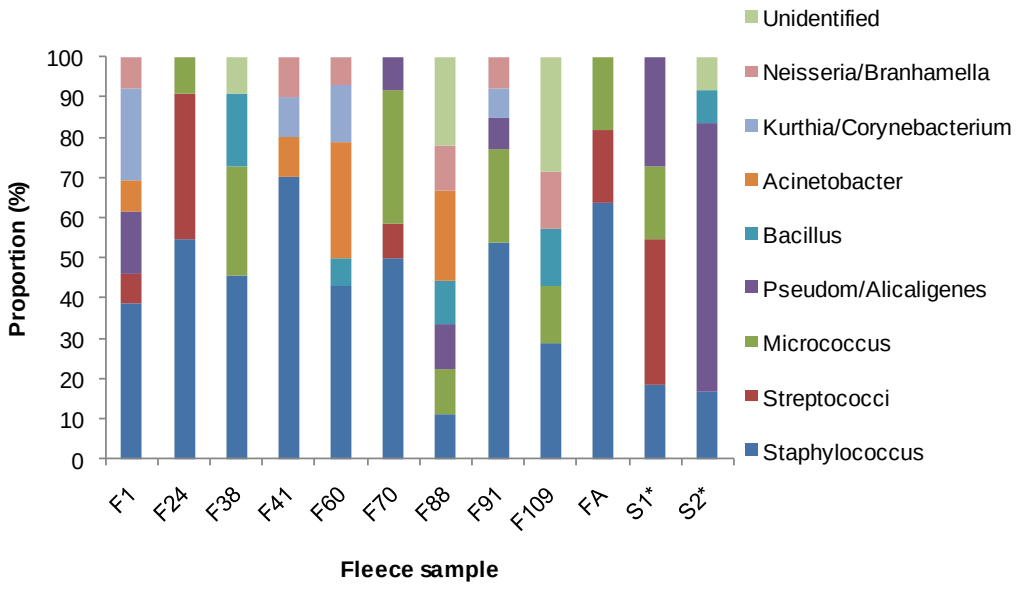

Figure 2.2 Bacterial community composition of healthy and scab-infected fleece. Each individual fleece sample divided into major bacterial groups as described by Cowan (2003), identified by biochemical tests. S1* and S2* are scab-infected fleece.

2.3.2 Isolation of Bacteria from *P. ovis* Faecal Trails

2.3.2.1 Identification with Biochemical Tests

When surface-sterilised mites from an *in vivo* culture (M1, Edinburgh) crawled over agar plates, lines of bacterial colonies were produced (Figure 2.3). The colonies differed in colour and morphology. Thirty colonies were picked, purified and following classification by biochemical and phenotypical tests (Appendix 1), ten distinct colonies (G16, G17, G18, G19, G22, G23, G24, G25, G26, G27) were picked from the isolates and classified by major bacterial group. Two were identified as *Micrococcus* (G19, G24), with others identified as *Staphylococcus* (G16), *Neisseria* (G17), *Alcaligenes* (G23), *Escherichia* (G25) and *Bacillus* (G26). Three were unable to be identified using biochemical methods (G18, G22, G27) (Appendix 6).

2.3.2.2 Sequencing of ITS region from *P. ovis* Faecal Bacteria

The ten mite faecal bacteria (MFB) were identified by molecular DNA sequencing, of which all had BLASTn maximum identity scores of greater than 71% and query

coverage of greater than 50% (except one of 47%) (Table 2.7). Of the ten, nine were identified as different bacterial species whereas two matched the same species (*Micrococcus luteus*). One bacterium (G18) was not able to be definitely identified by sequencing as the closest match was to an uncultured bacteria (Accession FM242723.1) and had a query coverage of 33%, which was below the threshold used (50%). There were species from three main bacterial phyla: Actinobacteria, Firmicutes and Beta-proteobacteria. Two samples most closely matched to 'uncultured bacteria', originally isolated from a wood boring beetle (*Anoplophora glabripennis)* and shown to have digestive functions for the beetle, so was classed as a symbiont (Geib *et al.*, 2009). Two of the MFB were classified as saprophytes, four as opportunistic pathogens and two unknown (Table 2.7).

The biochemical and DNA sequencing results were compared and six were identified as belonging to the same genus, G17, however, was identified as *Neiserria* using biochemical tests but *Carnobacterium* by molecular DNA sequencing (Table 2.7). Moreover, DNA sequencing was able to identify the bacteria to a higher taxonomic resolution than the biochemical tests, i.e. to a species level, therefore subsequent bacterial identification used molecular DNA sequencing. There were four genera present that were also present in fleece (healthy and scab-infected) (*Staphyloccus, Micrococcus, Bacillus* and *Pseudomonas*). There were, however, two novel genera (*Lactobacillus* and *Escherichia*) in this environment (Table 2.7).

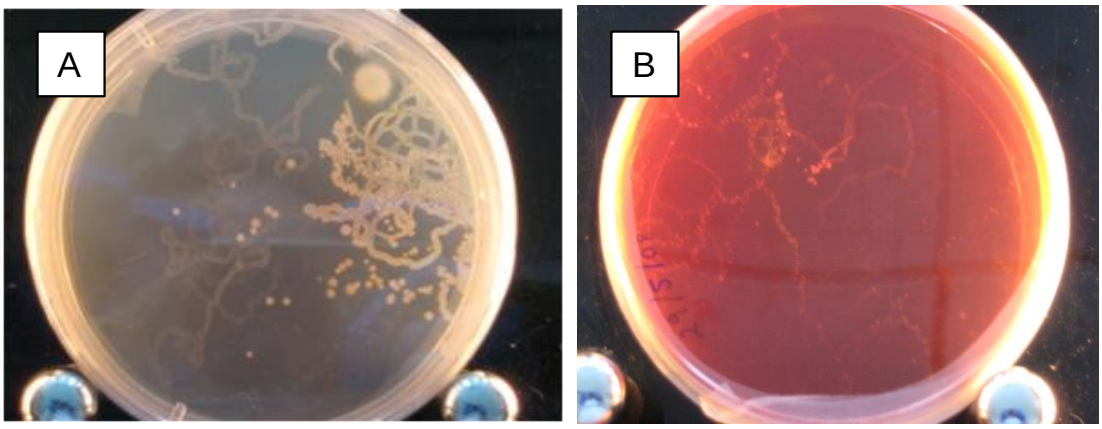

Figure 2.3 *P. ovis* faecal trails on agar. Bacterial growth from a single surface-sterilised M1 mite (*in vivo* culture, Edinburgh) on **A**: Lamb serum agar plate, **B**: MacConkeys agar.

Table 2.7 Identification of *P. ovis* faecal trail bacteria from closest BLASTn (http://blast.ncbi.nlm.nih.gov/Blast) match with accession numbers, maximum identity (%) and query coverage (%) values. Bacterial categories classified by biological characteristics: op. path., opportunistic pathogens, sap., saprophyte, path., strict pathogen, symb., symbiont. (**1**) Identification of bacteria using biochemical tests. One bacterium (G18) had matches below query threshold so could not be definitively identified by this method.

Phylum	MFB number	Closest BLASTn match (name, accession no,)	Max identity (%) (Query coverage %)	Bacterial category	Biochemical Classification[1]
Actinobacteria	G24	*Micrococcus luteus, AB088764.1*	82 (47),	sap./op.path	*Micrococcus*
	G19	*Micrococcus luteus, AB088764.2*	98 (60)	sap./op.path	*Micrococcus*
Firmicutes	G26	*Bacillus cereus, EU871042.1*	99 (100)	op.path	*Bacillus*
	G17	*Carnobacterium mobile AF374289.1*	71 (83)	sap.	*Neisseria*
	G16	*Staphylococcus aureus,* U39769.1	100 (59)	op.path	*Staphylococcus*
Beta-proteobacteria	G23	*Alcaligenes faecalis EU014606.1*	96 (90)	op.path	*Alcaligenes*
Gamma-proteobacteria	G25	*Escherichia coli FJ823387.1*	96 (55)	op.path	*Escherichia*
Uncultured bacteria	G22	Uncultured bacterium O1_44 FJ356614.1 (Geib *et al* 2009)	89 (74)	symb.	*Unidentified*
	G27	Uncultured bacterium O1_44 FJ356325.1 (Geib *et al* 2009)	95(68)	symb.	*Unidentified*

2.3.3 Use of PCR to Identify Bacteria Associated with Sheep Scab Disease

2.3.3.1 Bacterial Group-Specific Primers

Bacteria from *P. ovis* Samples

Single mites were surface sterilised and microlysed (Section 2.2.2.6) before specific-group PCR. Bacteria were detected in all mite samples (three from *in vivo* culture, M1, M2, M3; three from natural infections, S1, S4, S7, in duplicate) using the universal 16S rRNA bacterial primers (Fierer *et al.*, 2005)(data not shown). These six mite samples were then used for the group-specific PCR assays. Only Actinobacteria was successfully detected, suggesting the presence of this bacterium within the mites. Negative results were seen for *Firmicutes*, *Pseudomonas* and *Acetobacter*-specific PCR assays. As bacteria isolated from the *P. ovis* faecal trails (and identified by DNA sequencing, Table 2.7) had genera other than *Actinobacteria* present, it was decided to use molecular community analysis (ITS-PCR and DNA sequencing) for bacterial DNA from mite and fleece samples for taxonomic analysis (Section 2.3.3.2).

2.3.3.2 ITS PCR and RISA

Bacterial Communities of Fleece Samples using ITS-PCR

ITS-specific primers were run with extracted DNA from fleece (six healthy samples and 21 scab-infected fleece). Of these, all six healthy and 17 infected samples produced a range of band lengths on the agarose gel. This indicated a range of bacterial species present in the samples as each band represents an operational taxonomic unit (OTU) which varys with the length of the amplified ITS gene region. Samples were amplified in duplicate. Six samples (three healthy H24, H91, H109 and three scab-infected S9, S14, S23) (Figure 2.4) were then used for PCR clean up, transformation and DNA sequencing.

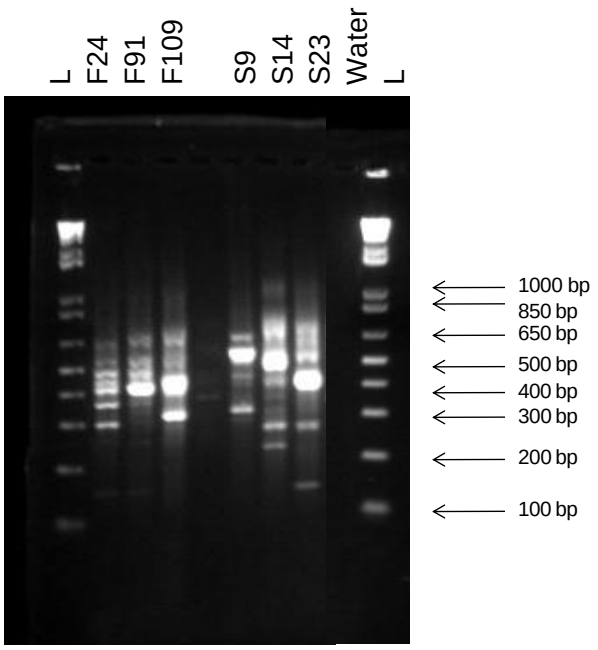

Figure 2.4 Amplification of DNA from healthy and scab-infected fleece using ITS-specific primers. Healthy (F) and Scab-infected (S) fleece samples, ITS PCR primers, ITSF and ITSReub (Fierer *et al.*, 2005), DNA standardised to 20 ng/µl. Includes water negative control and 1Kb^{+} ladder (L) for size calibration (Invitrogen).

Sequencing of ITS regions from Fleece Samples

From the six fleece samples that were sequenced (H24, H91, H109, S9, S14, S23), an average of 70 positive transformations (white colonies) from each sample were sub-cultured onto grid reference plates. They were checked for successful inserts and insert length with the M13-PCR, using bacteria directly from the grid plate (data not shown). From this, ten colonies, with varying band sizes, were sequenced from each sample. Cloning for sample S23 (scab-infected fleece) were repeatedly unsuccessful as all received sequences matched closest to vector sequences.

Sequence analysis of healthy fleece samples revealed four phyla, nine genera and 15 different bacterial species (by accession numbers) whereas infected fleece had representatives from three phyla, two genera and four different species (plus uncultured bacteria) (Table 2.8). This indicated the diversity of bacteria was greater in healthy fleece than scab-infected fleece in this study. This result confirmed the diverse composition of bacteria observed from the biochemical tests (Section 2.3.1.2). Healthy fleece had a wider range of bacterial types present than scab-infected, with six opportunistic pathogens, two pathogens, five saprophytes and one unknown (uncultured) (Table 2.8). Scab-infected fleece was dominated by opportunistic pathogens (three), one saprophyte and three unknown (uncultured) (Table 2.8).

Staphylococci sp. and *Bacteroidetes* sp. were commonly occurring bacteria as they were isolated from both healthy and scab-infected fleece. There were genera, however, that were restricted to one fleece type. *Pseudomonas* was only identified in the healthy fleece, which contrasts the microbiological fleece results (Section 2.3.1.2). In addition, healthy fleece had a number of Actinobacteria and Gamma-proteobacteria species that were not detected in scab-infected fleece in this study.

Comparing the biochemical and DNA sequencing results indicated some genera were identified through both methods from healthy fleece, such as *Staphylococcus* spp. (F24), *Pseudomonas* spp. (F91) and *Bacillus* spp. (F109). Unfortunately, different scab-infected fleece samples were used for biochemical tests and DNA sequencing, due to sample quantity, so cannot be compared in this way. This does, however, indicate the effects of different sampling and identification methods on bacterial community analysis and the higher resolution of taxonomic identification that culture-independent methods provide.

Table 2.8 Phylogenetic affiliation of ITS region sequences of bacteria from healthy and scab-infected fleece to closest matches in BLASTn database (http://blast.ncbi.nlm.nih.gov/Blast). Bacterial categories classified by biological characteristics: op. path., opportunistic pathogens, sap., saprophyte, path., strict pathogen. Figures given are maximum identity (%) and query coverage (%) respectively, of BLASTn match.

Phylum	Genera	Bacterial categories	Max identity (%) (Query coverage %)				
			Healthy			Scab-infected	
			F24	F91	F109	S9	S14
Actinobacteria	*Corynebacterium sp.* BX248360.1	op. path.	88 (54)				
	Micrococcus luteus AB088764.1	sap.		80 (100)			
	Nocardia beijingensis GQ853482.1	op. path.		95 (95)			
	Rathayibacter tritici AY191505.1	path.		76 (90)			
	Tropheryma whippeli, AJ551273.1	path.		100 (74)			
	Bacteroides fragilis GQ496394.1	sap.	90 (80)		90 (64)		91 (64)
Firmicutes	*Bacillus sp* AB243783.1	sap.	94 (62)	94 (56)			
	Bacillus fusiformis AF478083.1	sap.			94 (60)		
	Staphylococcus aureus, AF478083.1	op. path.			94 (60)		
	Staphylococcus sp. AY728162.1	op. path.	94 (79)				88 (73)
	Staphylococcus aureus U39769.1	op. path.					87 (68)
	Staphylococcus xylosus U39773.1	op. path.	86 (69)				91 (59)
Gamma-Proteo bacteria	*Pseduomonas sp DQ003234.1*	op. path.	74 (78)				
	Pseudomonas chloroaphis DQ023306.1	sap.		100 (65)			
	Pseudomonas stutzeri U65012.1	op.path	79 (66)				
Uncultured	Uncultured 70113 AY484712.1	-				96 (57)	
	Uncultured bacterium AB222629.1	-				81 (73)	
	Uncultured Pseudomonadales AB491964.1	-				90 (71)	
	uncultured t1010 AF422501.1	-	70(80)				

RISA of Fleece Bacterial Communities

DNA extracted from fleece samples was amplified using the ITS-PCR as previously described (Section 2.2.2.10) then PCR products were separated automatically according to size by the Agilent 2100 Bioanalyzer (Agilent Technologies).

As seen on the agarose gel (Figure 2.4) the electronic output of the Bioanalyzer indicated the microbial communities of fleece were complex with different numbers of bands present in each sample (Figure 2.5). There appeared to be similar banding patterns between the agarose gel and bioanlayser output, for example the two bands at approximately 300 bp and 400 bp in the two samples of F109, whereas some samples were more different (for example S14) (Figure 2.4, 2.5).

From this a bottom-up clustering method was used to produce a dendrogram by unweighted pair group method with arithmetic mean (UPGMA). This compared the similarity and diversity between samples using the Jaccard's coefficient (Jaccard, 1912). This analysis revealed that the microbial communities associated with scab-infected fleece were not strictly defined by time or location as clustering by these factors was not seen (Figure 2.6). Extracted DNA samples were standardised to 20 ng/µl before PCR and the relative abundance of different bacteria (OTU) varied within samples as indicated by bands with different weights, which directly correlates to electropherogram peak size (data not shown).

Except for three samples from Edinburgh February 2008 (F41, F1, F25) which clustered together the rest of the healthy and scab-infected fleece samples did not show any clustering patterns due to fleece type (Figure 2.7), which indicates individual samples are have similar levels of complex ity, with a number of different banding patterns (OTU) present as seen with both the biochemical and DNA sequencing results (Section 2.3.1, 2.3.3.2). This analysis allows for bacterial communities between samples to be compared, yet does not identify bacterial species present.

The number of bands present in the fleece RISA profiles (27 scab-infected and 8 healthy samples) was compared using a T-test. There was no significant difference between the number of OTU bands observed between healthy and scab-infected (T_{34}=0.84, P = 0.405), indicating there was no difference in level of diversity within the samples. There was also no significant difference between minimum and maximum band lengths (T_{34} =0.16, P =0.877, $T_{27.7}$ = -0.41, P = 0.684 respectively), or in the range of bands observed between samples ($T_{25.8}$ = -0.48, P = 0.637) in RISA profiles.

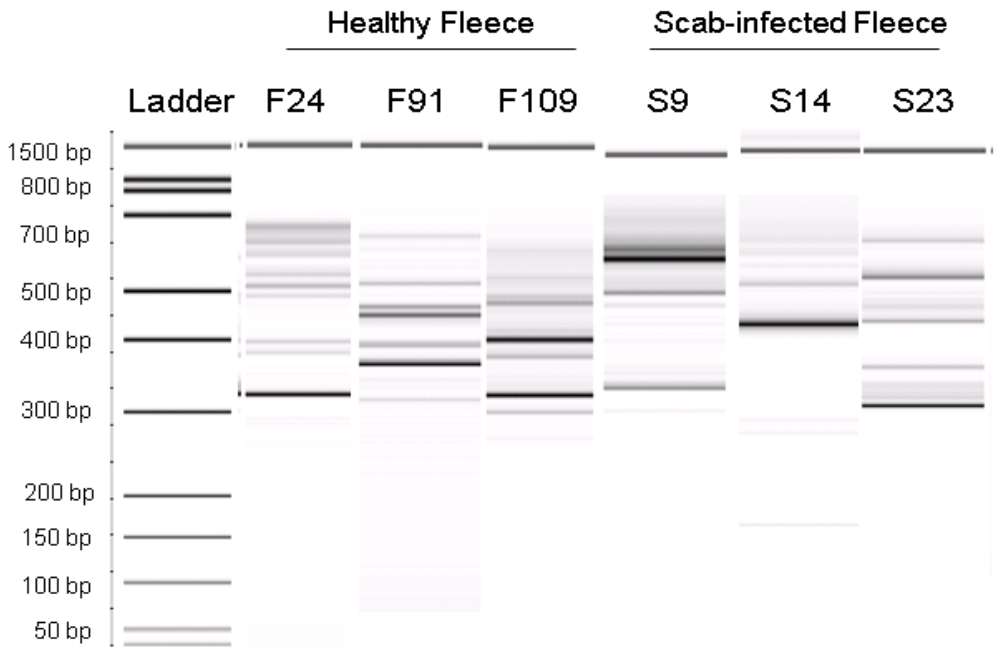

Figure 2.5 Amplification of DNA from healthy and scab-infected fleece using ITS-specific primers and analysed with Bioanalyzer (Agilent). Ladder: 50-1500 bp molecular ladder for size calibration. Each band represents an operational taxonomic unit (OTU) (different bacterial species) and the weight of the bands correlates to the size of the electropherogram peak produced which indicates the abundance of the different OTU present.

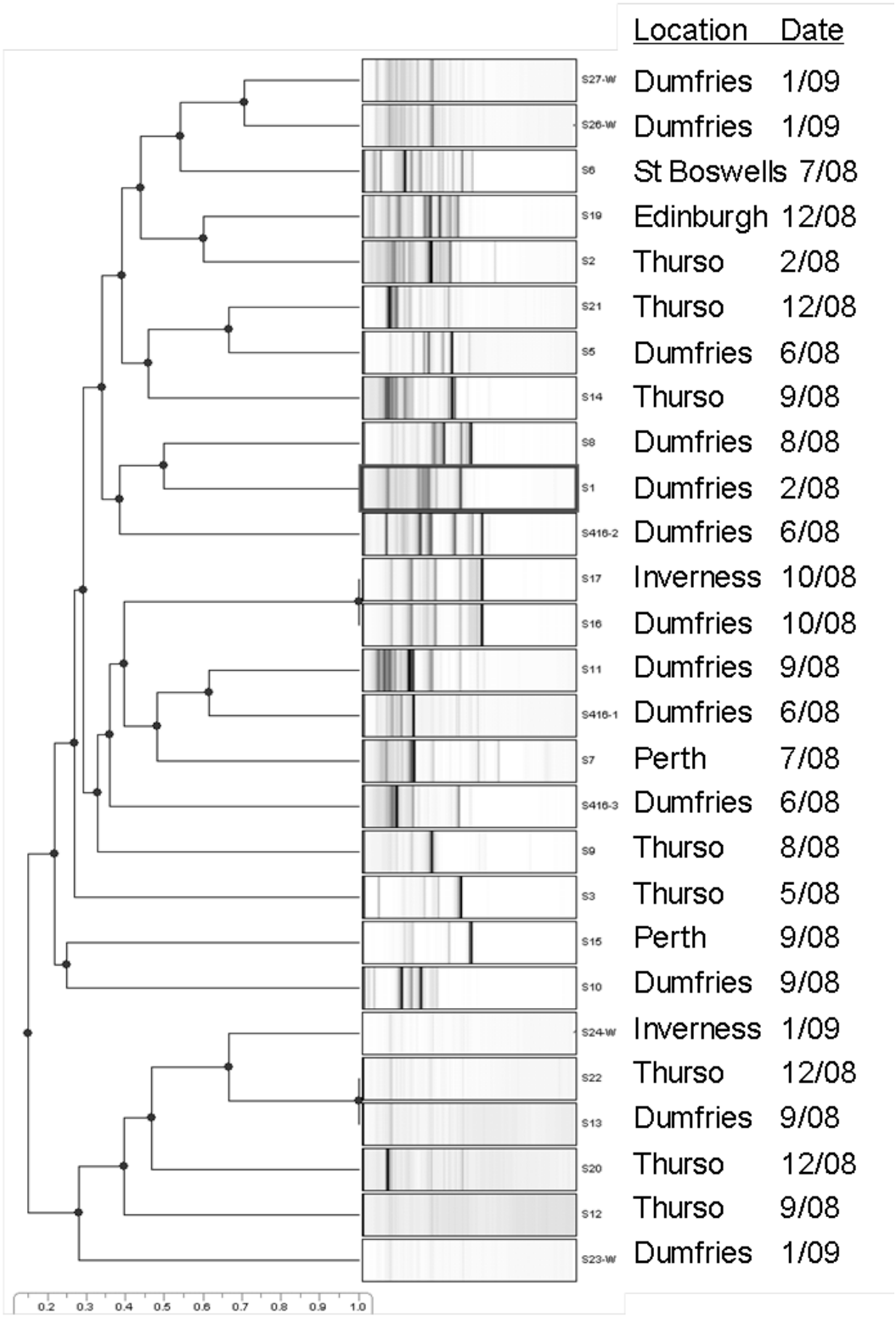

Figure 2.6 Dendrogram of RISA for all scab-infected fleece samples. Clustered by UPGMA (unweighted pair group method with arithmetic mean) using TotalLab. Geographical location and date received shown to right. Scale is Jaccard's coefficient. There was no clustering by either date or location

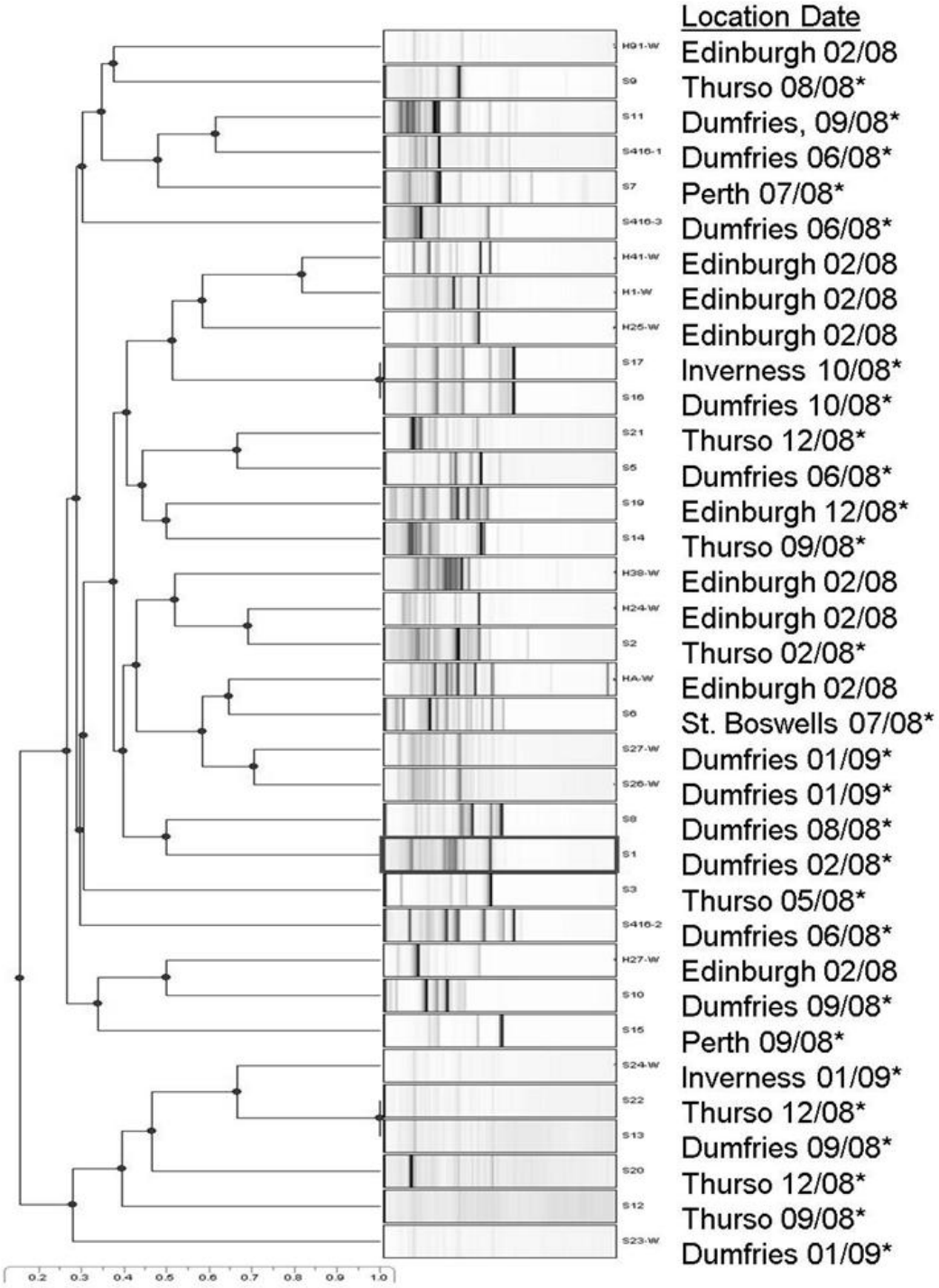

Figure 2.7 Dendrogram of RISA for all scab-infected (*) and healthy fleece samples. Clustered by UPGMA (unweighted pair group method with arithmetic mean) using TotalLab. Scale is Jaccard's coefficient. Scattering of samples indicates similar bacterial species in both fleece types.

Bacterial Communities of *P. ovis* Samples by ITS PCR

DNA was extracted from *P. ovis* mites (three *in vivo* cultures and eleven natural sheep scab infections) and amplified using the ITS-specific primers. As seen with the fleece (Section 2.3.3.2), a number of bands representing OTUs were produced from each mite sample indicating a number of different bacterial species present. As for the fleece samples, three of the mite samples from both the *in vivo* mite culture (M1, M2, M3) and the natural scab infections (S193, S21, S22) (Figure 2.8) were selected for PCR clean up, transformation and sequencing.

Sequencing of ITS regions from *P. ovis* DNA Samples

In total 126 plasmid preparations were produced, of which 72 (57%) produced clear bands with the M13 PCR, which comprised 5, 15 and 1 from M1, M2 and M3 respectively and 10, 25 and 16 from S193, S21 and S22 respectively. These 72 plasmids were sent for sequencing and of these, 54 (75%) from all samples were successfully sequenced and matched using the BLASTn database from both sample types. There were 1, 9 and 8 samples from M2, S21 and S22 respectively, which did not return any data after sequencing.

The bacteria identified from *P. ovis* samples had representatives from five phyla: Actinobacteria, Firmicutes, Bacteroidetes, Beta-, and Gamma-proteobacteria as well as some unidentified uncultured bacteria (Table 2.9). Overall, bacteria isolated from the *in vivo* cultured mites contained seven opportunistic pathogens, two pathogens, three saprophytes and two unknown. The natural infection mite samples, however, comprised of five opportunistic pathogens, one pathogen, three saprophytes, one unknown and one arthropod symbiont (Table 2.9).

Whole mite extracts included species previously isolated from *P. ovis,* such as *Propionibacterium acnes* and *Staphylococcus chromogenes*. There were a number of bacteria isolated from *P. ovis* for the first time, including *Acinetobacter iwoffii, Moraxella osloensis, Pseudomonas fragi, Vibrio alginolyticus, Psychrobacter* sp.*, Comamonas testosteroni, Janthinobacteria* sp. and uncultured V*errucomocrobia* (Table 2.9). *Staphylococcus xylosus* and *Vibrio alginolyticus* were isolated from all the natural infection mite samples, indicating a common bacterial species irrespective of geographical origin. *Comamonas testosteroni* and *Moraxella osloensis* have are symbionts of arthropods (Zouache *et al*., 2009) and nematodes (Tan & Grewal, 2011) respectively. Eleven different species were isolated from both *in vivo* cultures and

natural mite samples, however, there were some differences in the species composition (Table 2.9). *In vivo* cultured mite samples had a greater variation of *Bacillus* and *Pseudomonas* species compared to the natural samples. Moreover, *Actinobacteria* spp. were only detected in the *in vivo* cultured samples (Table 2.9).

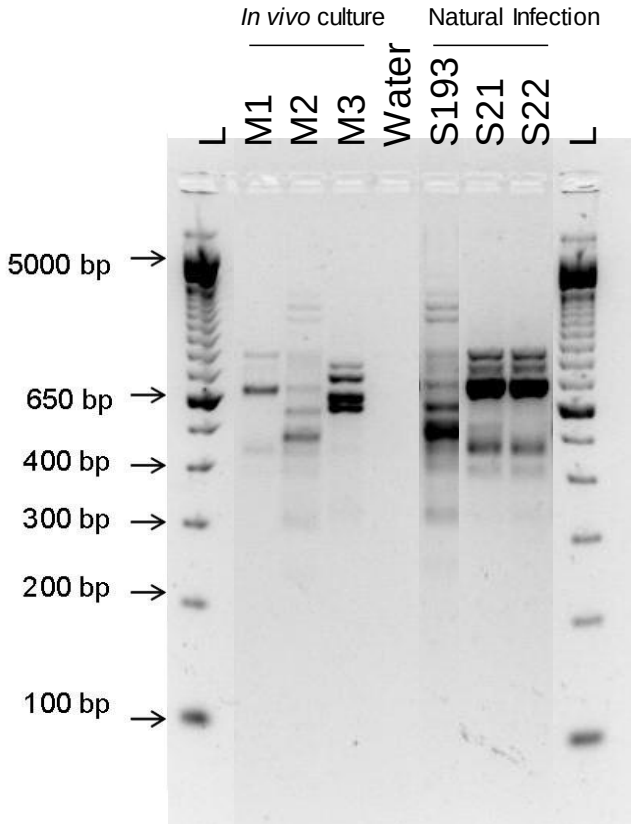

Figure 2.8 DNA extracted from *P. ovis* mite samples, amplified with ITS-specific primers. Samples: *in vivo* cultured (M1, M2, M3) and natural infections (S193, S21, S22), primers ITSF, ITSReub (Fierer *et al.*, 2005) L: 1 Kb^{+} ladder for size calibration (Invitrogen), Water: water negative control. Each band represents an operational taxonomic unit (OTU).

Table 2.9 Phylogenetic affiliation of ITS sequences of bacteria from *P. ovis* mites to closest matches in BLASTn database (http://blast.ncbi.nlm.nih.gov/Blast). Bacterial categories classified by biological characteristics: op. path., opportunistic pathogens, sap., saprophyte, path., strict pathogen, symb., symbiont. Figures given are maximum identity (%) and query coverage (%) respectively of BLASTn match.

		Max identity (%) (Query coverage %)					
		in vivo cultured mites			Natural infection mites		
Closest BLASTn match (name, accession no,)A	**Bactrial categories**	**M1**	**M2**	**M3**	**S193**	**S21**	**S22**
Corynebacterium amycolatum ,	*op. path.*		87 (68)				
Propionibacterium acnes,	*op. path.*			88 (67)			
Tropheryma whipplei,	*path.*		100 (98)				
Bacillus cereus, EU915688.1	*op. path.*	100 (80)	100 (100)				
Bacillus cereus, GQ255884.1	*op. path.*	100 (81)					
Bacillus thuringiensis,	*sap.*	98% (98%)			98% (90%)		
Staphylococcus aureus,	*op. path.*				89% (72%)		
Staphylococcus	*op. path.*					98% (66%)	97% (66%)
Staphylococcus epidermidis,	*op. path.*					77 (68)%	
Staphylococcus hyicus,	*op. path.*						92% (63%)
Staphylococcus xylosus,	*op. path.*				90% (66%)	88% (72%)	
Comamonas testoreroni,	*symb.*					97% (78%)	
Acinetobacter genomosp,	*sap.*		79% (86%)				
Acinetobacter iwoffii,	*sap.*					93%(56%)	
Klebsiella oxytoca,	*op. path.*		98% (88%)				
Moraxella osloensis,	*symb.*					91% (70%)	
Pseudomonas sp,	*op. path.*		97% (92%)				
Pseudomonas putida,	*sap.*		100% (74%)				
Pseudomonas mendocina,	*op. path.*		94% (91%)				
Vibrio alginolyticus,	*path.*		86% (74%)		86% (75%)		78% (82%)
Xanthomonas sp,	*sap.*						99% (83%)
Uncultured verrucomicrobia, AM279407.1	-				92% (51%)		
Uncultured bacterium S10-2	-	94% (90%)					
Uncultured bacterium O1_44	-		84% (90%)				
Uncultured bacterium TIM15-4,	-						92% (79%)

RISA of *P. ovis* Mite Bacteria Communities

In a similar way to the fleece (Section 2.3.3.2), extracted DNA from mites was amplified using ITS-PCR and then compared using the Bioanalyzer (Agilent). The profiles, however, were less distinct than the fleece samples and fewer bands were observed compared to the mite gel, especially with samples M1 and S193 (Figure 2.9).

When RISA profiles of mites from the *in vivo* culture (3 samples) were compared with mites received from natural sheep scab infections (3 samples), no significant difference was seen for the number of OTU bands in the profile (median 3.5 for both) ($U_{6,6}$=15, P=0.699), minimum OTU band size (median 211.6 bp, 394.5 bp for *in vivo* and natural infection respectively)($U_{6,6}$=8, P=0.132), or maximum OTU band size (median 651.5 bp *in vivo* and 842.3 bp natural infection) ($U_{6,6}$=12, P=0.394). This indicated that mites from *in vivo* cultures and natural infections had similar frequencies of different bacteria present. When the *P. ovis* RISA profiles were compared with a dendrogram (Figure 2.10) clustering of replicates could be seen, in addition to clustering by geographical origin and date, which may indicate that mite bacterial communities are affected by their geographical origins.

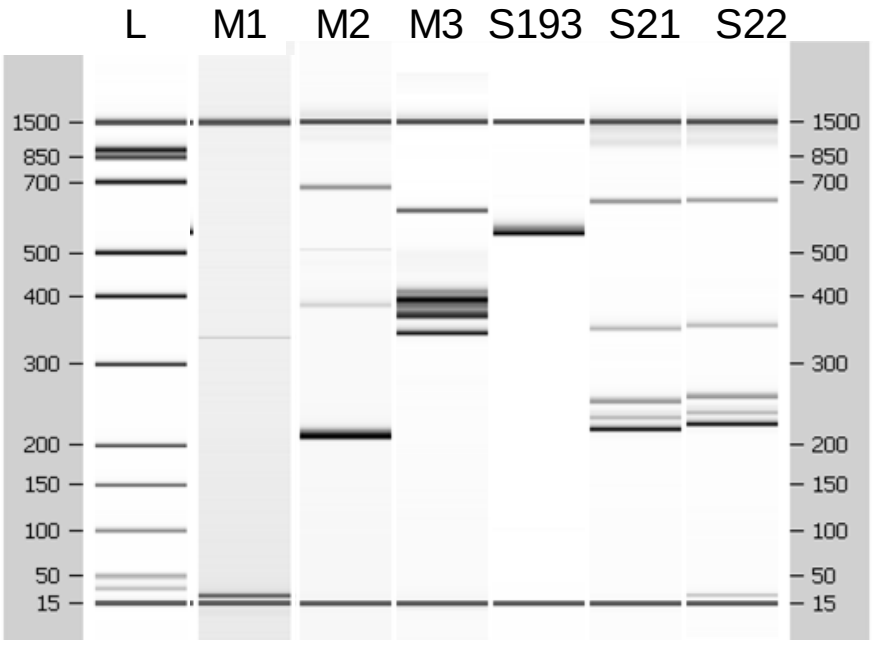

Figure 2.9 RISA output for *P. ovis* samples analysed with Bioanalyzer (Agilent). Samples: M1-3 *in vivo* culture, S193-S22 natural samples. L: 15-1500 bp molecular ladder for size calibration. Each band represents an operational taxonomic unit (OTU) (different bacterial species) and the weight of the bands correlates to the size of the electropherogram peak produced which indicates the abundance of the different OTU present.

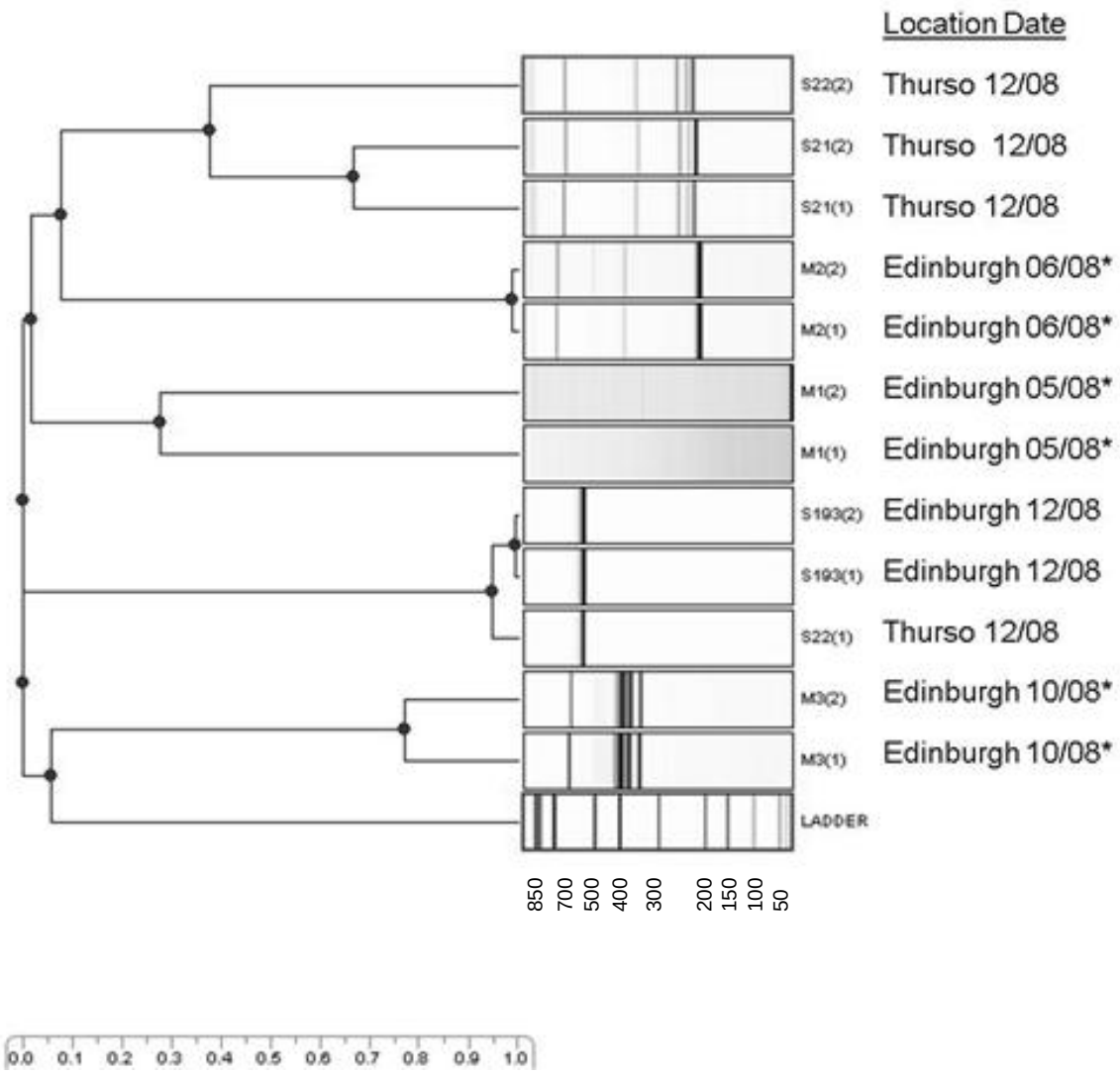

Figure 2.10 Dendrogram of RISA profiles for *in vivo* culture (M*) and natural infection (S) of *P. ovis* samples. Clustered by UPGMA (unweighted pair group method with arithmetic mean) using TotalLab. Replicate (n=2) number in brackets after sample number. Geographical location and date received shown to right. Scale is Jaccard's coefficient, ladder in basepairs. Clustering by date and location is seen.

2.3.3.3 Summary of Results: Bacteria Associated with Sheep Scab Disease

Molecular DNA sequencing of the bacterial ITS region revealed a number of bacterial genera associated with sheep scab disease. Pooling individual samples from each source (whole crushed *P. ovis, P. ovis* faceal trails and fleece) indicated much variation among sample types (Table 2.10). There were six common bacterial species between healthy fleece and sheep scab-derived samples including *Micrococcus luteus*, *Tropheryma whipplei*, *Staphylococcus* sp., *S. aureus*, *S. xylosus* and *Pseudomonas* sp. Within sheep-scab derived samples, there were five common species including *B. cereus*, *B. thuringiensis*, *S. aureus*, *S. xylosus* and *Vibrio alginolyticus*. These differences in bacterial presence may indicate changes in bacterial communities coinciding with sheep scab disease infection.

Table 2.10 Summary of bacteria identified in this study using ITS-specific primers. Samples included: *P. ovis* mites, *P. ovis* faecal trails, healthy and scab-infected fleece. Individual sample ID is given with closest BLASTn species match. X indicates presence of bacteria within sample type.

		Source of Samples				
		Sheep fleece-Healthy	**Sheep fleece Scab-Infected**	***P. ovis* gut trail**	***P. ovis*, natural infection**	***P. ovis*, *in vivo* culture**
	Sample ID	F24	S9	M1	M1	S193
		F41	S14		M2	S21
		F109	S23		M3	S22
Phylum	**Closest BLASTn species match**					
Actinobacteria	*Corynebacterium amycolatum*					x
	Corynebacterium diphtheriae	x				
	Micrococcus luteus	x		x		
	Nocardia beijingensis	x				
	Propionibacterium acnes					x
	Rathayibacter tritici	x				
	Tropheryma whippeli	x				x
	Bacteroides fragilis	x	x			
Firmicutes	*Bacillus* sp	x				
	Bacillus cereus			x		x
	Bacillus fusiformis	x				
	Bacillus thuringiensis				x	x
	Carnobacterium mobile			x		
	Staphylococcus sp	x	x			
	Staphylococcus aureus	x	x	x	x	
	Staphylococcus chromogenes				x	
	Staphylococcus hyicus				x	
	Staphylococcus xylosus	x	x		x	
B-proteo-bacteria	*B-proteobacteria*			x		
	Comamonas testosteroni				x	
Gammaproteobacteria	*Acinetobacter genomosp*					x
	Acinetobacter iwoffii				x	
	Escherichia coli			x		
	Klebsiella oxytoca					x
	Moraxella osloensis				x	
	Pseudomonas sp	x				x
	Pseudomonas chlororaphis	x				
	Pseudomonas mendocina					x
	Pseudomonas stutzeri	x				
	Vibrio alginolyticus				x	x
	Xanthomonas sp				x	
Uncultured bacteria	Uncultured bacterium	x	x	x	x	x

2.3.3.4 Screening of *P. ovis* Mites using Endosymbiotic Bacterial Primers

Mite samples (eight *in vivo*, M1-M8; six natural infection, S1, S11, S19, S21, S22, S29/5) were amplified with endosymbiont-specific primer sets (Section 2.2.2.7). This was the first known screening of *P. ovis* mites for the presence of endosymbiotic bacteria.

Of four endosymbiont PCR assays, positive bands from *P. ovis* mites were produced for *Cardinium* (4/14) (29%) and *Comamonas* (6/14) (43%), whereas all mite samples were negative for *Rickettsia* and *Wolbachia*. It was not possible to source positive controls, except for *Wolbachia* DNA, for endosymbiont-specific PCR; instead *D. radicum* samples were used to optimise PCR reactions because this organism harbours endosymbiotic bacteria (Cordaux *et al.*, 2008). For this reason, positive bands from PCR were excised from the gel, purified and sequenced to confirm endosymbiont DNA presence.

Following sequencing, DNA amplified with *Cardinium* primers (CHF/CHR) from Zchori-Fein & Perlman (2004) matched using BLASTn to *Comamonas testosteroni* strains (accession HQ200412.1, FJ426595.1) with 98% and 97% max identity and 100% and 97% coverage, respectively, indicating the *Cardinium* primers also amplified *Comamonas* spp. In light of this, *P. ovis* mites were only positive in this study for the known arthropod-endosymbiont bacterium *Comamonas* sp. and it was present in both *in vivo* laboratory cultures and natural mite populations in this study (Table 2.11).

Table 2.11 Screening of *P. ovis* mites with endosymbiont-specific primers. PCR results were positive (**+**) or negative (**-**), all were run in duplicate and confirmed by DNA molecular sequencing using 16S rRNA-specific primers. *P. ovis* mites from both *in vivo* cultures and natural infections were tested in addition to *Delia* spp. flies for additional control samples.

Sample	*P. ovis* Origin	PCR Endosymbiont assay			
		Cardinium	*Comamonas*	*Wolbachia*	*Rickettsia*
M1	*in vivo* culture	-	+	-	-
M2		-	+	-	-
M3		-	+	-	-
M4		-	-	-	-
M5		-	-	-	-
M6		-	-	-	-
M7		-	-	-	-
M8		-	-	-	-
S1	Natural infection	-	+	-	-
S11		-	-	-	-
S19		-	-	-	-
S21		-	+	-	-
S22		-	+	-	-
S29/5		-	-	-	-
D. radicum	Flies	+	+	+	+
D. coarctata		-	-	-	-

2.4 Discussion

2.4.1 Bacteria Identified from Fleece and Mites

It is apparent from this study that the microbial flora associated with sheep scab disease is very complex. Bacterial density in healthy fleece was higher than in infected fleece. Bacterial density was measured on five different agar types to allow for the growth of a broad range of bacterial species, however, only healthy fleece samples produced bacteria that grew on MRS agar, which supports the growth of lactobacillus bacteria. There was no growth on MRS agar from scab-infected samples, which is interesting as this agar favours lactobacilli, which were detected in these samples by molecular DNA sequencing. One reason for this could be the slow rate of growth characterised by this bacterial group (Bridson, 1990). This may indicate a change in composition or loss of these bacteria from healthy fleece upon scab infection. There may have been bacterial competition on the plates, affecting growth of other bacterial species (Torsvik *et al.*, 1990). The bacterial density for healthy fleece in this study (median Log5 CFU/g) was slightly higher than previous studies of healthy fleece, Gochel *et al.,* (1992) recorded Log3-4 mean CFU/g healthy fleece, yet within the range observed in microbial density which can vary with the location of the fleece origin from inner (Log4.6 CFU/g) or outer (Log7.3 CFU/g) staple of fleece (Jackson *et al.*, 2002). Although the exact origin of scab-infected fleece samples was unknown in this study, healthy fleece was taken from close to the skin.

Culture-based and molecular results indicated that bacteria present in healthy fleece were very diverse, with fifteen different species identified in this study. The majority of bacteria identified within this environment were from the Firmicutes and Gamma-proteobacteria phyla. Overall, healthy fleece had a greater diversity of bacterial species than scab-infected fleece but this could be due to a higher transformation success rate.

Microbiological techniques (biochemical and phenotypic tests) revealed that *Staphylococci* and *Micrococcus* species were dominant on healthy fleece whereas scab-infected fleece was dominated by *Pseudomonas* species, although *Pseudomonas* was also present on healthy fleece. These results corroborate previous work on sheep fleece bacteria (Merritt & Watts, 1978a; Murray & Edwards, 1987; Chin & Watts, 1992; Lyness *et al.*, 1994) and other ectoparasite infections of sheep, *Bovicola ovis* (Murray & Edwards, 1987; Oliveira *et*

al., 2006), *P. cuniculi* (Bjotvedt & Geib, 1981), mange mites on cattle (Hazarika *et al.*, 1991) and sarcoptes mites on pigs (Hogg & Lehane, 1999).

The fleece used in this study was from Blackface, Suffolk and crossbreeds which are common Scottish sheep breeds (Ryder, 1964; Carlyle, 1979). Due to differences in fleece composition between breeds and associated bacterial communities (Meyer *et al.*, 2001), conclusions from this study are restricted to these breed types. Moreover, little information is available on fleece variation between individuals (Dixon *et al.*, 2007), as this study concentrated on the differences in species composition between healthy and scab-infected fleece. There will, however, also be inherent variation in bacterial composition of fleece due to other factors including: environmental factors such as incidence of rain which can change fleece bacterial composition (Merritt & Watts, 1978b), variation in fleece attributes, such as resistance to fly strike (Raadsma *et al.*, 1988) as well as temporal factors.

The dominant species detected on fleece in this study are common soil and sheep-skin bacteria (Meyer *et al.*, 2001). Identification of bacteria by molecular sequencing of scab-infected fleece was less successful than for the other samples (mites and healthy fleece), although the reason for this is unclear. It was surprising that *Pseudomonas* was not isolated by DNA sequencing of bacteria isolated from scab-infected fleece, the reason for this could be sampling technique or preferential amplification of other bacteria in the PCR reaction (Sommaruga & Casamayor, 2009). It may also be due to deterioration of the scab-infected fleece before analysis owing to the extended time period before receipt of sample.

During development of sheep scab disease, the skin may be weakened by wetting (serum exudation) and bacterial exoenzymes (Lyness *et al.*, 1994) through which bacteria contribute to the pathogenicity of the disease (Bates, 2003). In addition, *P. ovis* may play a part in the transmission and replication of bacteria on the skin of the sheep (Bates, 2003). It has been shown that bacteria play a critical role in the development of non-parasitic disease as well, such as fleecerot (Baird, 2000), which involves many bacterial species similar to those isolated from *P. ovis* in this study including *Corynebacterium* sp., *Escherichia coli, Acinteobacter* sp.*, Moraxella osloensis* and *Pseudomonas* sp. (Dixon *et al.*, 2007).)

Hogg and Lehane (2001) concluded that there was no single bacterium associated with *P. ovis* mites, unlike other arthropods which can be dominated by one bacterium, such as

tsetse fly and its symbiont, *Wigglesworthia* (Aksoy, 2000). This study found further evidence of diverse bacterial species to support this statement as well as identifying a number of bacteria not previously identified in *P. ovis*, including *Acinteobacter* sp., *Moraxella* sp, *Pseudomonas fragi*, uncultured *Verrucomicrobia* and *Comamonas* sp.

Serratia marcescens was not identified from any samples in this study despite having previously been described as being 'the most isolated bacterium from *P. ovis*' (Mathieson & Lehane, 1996; Perrucci *et al.*, 2005). Studies that have previously isolated *S. marcescens* used mites cultured *in vivo*, as opposed to natural infections (Mathieson & Lehane, 1996; Hogg & Lehane, 2001). *S. marcescens* has been noted to colonise laboratory cultures of arthropods (Azambuja *et al.*, 2004) and even become pathogenic to arthropod hosts (Grimont & Grimont, 1978). The hypothesis of this bacterium being beneficial to mites was deemed unlikely as a result of selective removal experiments with *P. cuniculi* which resulted in no discernable affect on mites' infective ability (Perrucci *et al.*, 2005). Therefore it is likely that this bacterium was a laboratory contamination and is not widely present in natural *P. ovis* mites as a beneficial organism.

Bacillus cereus and *Staphylococcus aureus* were identified from both faecal trails and whole mite extractions and *Micrococcus luteus* and *S. aureus* were isolated from both faecal trails and fleece. This indicated that these bacteria may be transient or dynamic through *P. ovis* and are components of the excreted faecal pellets. Bacteria isolated from *P. ovis* faecal trails are indicative of bacteria that are transmitted to the skin of sheep and potentially encountered by other mites. No conclusion about their role within *P. ovis* can be drawn from this chapter. The associations of bacteria to *P. ovis* is addressed in Chapter 3.

This study showed that there was heterogeneity in the composition of bacterial populations associated with natural and *in vivo* cultures of *P. ovis*, as shown previously by Hogg & Lehane (2001). *In vivo* mites in this study were maintained on sheep housed in indoor enclosures. This could therefore affect the diversity of bacteria the sheep would encounter as compared to hill-dwelling sheep similar to the origin of the natural mite samples (Table 2.4). The decrease in diversity associated with laboratory-rearing of organisms is widely reported (Hogg & Lehane, 2001).

It has been observed that the bacterial flora of *P. ovis* differs greatly with geographical distribution, that virulence of this disease varies and that the two factors may be linked (Mathieson & Lehane, 1996; Hogg & Lehane, 2001). This could not be investigated in

this study due to the lack of virulence data associated with mite samples. This highlights the importance of studying geographical differences in the future.

There were several bacteria identified in *P. ovis* that are known opportunistic pathogens of animals, such as *Propionibacterium acnes* (Hogg & Lehane, 2001), *Pseudomonads* (Chin & Watts, 1992), *Bacillus* and *Staphylococcus* species (Bisset, 1962), all of which may enhance the pathogenicity of sheep scab disease (Merritt & Watts, 1978a; Bates, 2003). *P. acnes*, which favours anaerobic conditions, has a known lipase action on sebum triglycerides (Namazi, 2007) which are present on sheep skin. The allergic dermatitis exhibited in sheep scab is caused by guanine-rich faecal pellets (Lewis, 1997). Moreover bacteria associated with sheep scab may also be potential sources of toxins that increase the pathogenicity of the disease. House dust mites (*Dermatophagoides pteronyssinus*) carry *Bartonella* among other Gram negative species, which have been suggested to be the source of endotoxins found in the mite allergenic extracts (Valerio *et al.*, 2005). In addition, proteinases have been isolated from *P. ovis* that have the ability to initiate lesions and degrade sheep proteins, potentially assisting mite feeding (Kenyon & Knox, 2002; Hamilton *et al.*, 2003). Some arthropod hosts with endosymbiotic bacteria, such as ticks, secrete saliva, which contain chemicals for digestion (Douglas, 1998; Graf *et al.*, 2006). It was previously thought that *P. ovis* do not possess salivary glands (Kirkwood, 1986), however, recent work has isolated a homolog saliva protein from *P. ovis* (McNair *et al.*, 2010).

Bacillus spp and *Staphylococcus* spp were the most numerous in isolations, both in terms of species richness (number of different species) and across sample types. The isolation of *E. coli* from faecal trails was interesting as although it is a common faecal bacteria among organisms (Hancock *et al.*, 1997; Johnson *et al.*, 2004) it has not been previously associated with *P. ovis* mites, yet this bacterium has been isolated from diseased sheep fleece (Tadayon *et al.*, 1980) and fleece from sheep resistant to fly strike (Dixon *et al.*, 2007). Although ubiquitous, this bacterium has high levels of genetic diversity (Johnson *et al* 2004) indicating its adaptations to different niches. Species identified were composed of bacteria from the proteobacteria class, but only the beta and gamma phyla were represented. Some bacteria were only isolated from one sample type such as *Corynebacterium amycolatum, Propionibacterium acnes* and *Tropheryma whipplei.* There are a

number of potentially interesting functions/roles that have been shown to be provided by bacteria isolated from mites (Appendix 7).

Abundance of each bacterial species present within *P. ovis* or fleece cannot be concluded from this study due to the sampling technique, which involved selection of colonies based on differing sequence length. An exhaustive search would be required to fully investigate this, but this was not financially possible in this study.

In total, ten bacteria were isolated from faecal trails, of which, nine were unique species. The differences are not surprising as initial cultures were chosen by their differing colony morphology. A few of the species were bacteria associated with faecal and animal skin habitats (*S. aureus, E. coli, M. luteus*). One species, *A. faecalis* within the *Pseudomonas/alcaligenes* group has been previously isolated from orbatid mites (Smrz & Norton, 2004) and poultry red mites (Moro *et al.*, 2009; De Luna *et al.*, 2009). There were two bacteria that most closely matched against 'uncultured bacteria' isolated from the gut of a wood-boring beetle, where it has a digestive and nutrient provisioning function (amino acids, vitamins and lipids) (Geib *et al.*, 2009). *Carnobacterium* was the only lactobacilli isolated from faecal trails. Although there is little published about this bacteria, it has be classed as allochthonous (transient) that are unable to colonise the gut except in rare circumstances (Dillon & Dillon, 2004).

The faecal trails were all carried out using *in vivo* cultured mites as the natural samples were often too desiccated, or too few present to carry out the experiment. It would be essential, however, to compare faecal trails between natural and *in vivo* strains of mite to observe differences in excreted bacteria between these environments.

2.4.1.1 Endosymbionts in *P. ovis*

This is the first known time *P. ovis* mites have been screened for bacteria known to be endosymbionts in other arthropods. Of six arthropod-specific-endosymbiont assays, only the *Comamonas*-specific assay was positive for *P. ovis* mites. This bacterium was detected in both *in vivo* cultured mites and natural samples, in addition to whole-mite ITS-PCR analysis. It is interesting that the positive *in vivo* cultured mites were from consecutive samples (M1-M3), as they make up a connected, continuous population over time. The *in vivo* culture is maintained by transferring mites from infected to naïve

sheep. It is unknown, however, whether M1 individuals harboured this bacterium and subsequently transferred it to naïve mites in the new population or whether each 'infection' with *Comamonas* was independent. *Comamonas* was also detected in natural samples of mites (S21, S22), indicating the incidence of this bacterium may not be confined to laboratory cultures and may therefore be much more widespread in *P. ovis* populations. *Comamonas* may be transovarially transmitted by a number of mechanisms: passive contamination of eggs with bacteria, probing of mothers faeces by offspring (proctophagy) or capsule transmission, where bacteria are deposited directly into egg capsule (Lam *et al.*, 2007). Bacteria could also be horizontally transmitted to other mites when feeding and excreting on the sheep body surface (Duron *et al.*, 2008; Zouache *et al.*, 2009b).

Comamonas spp. has previously been detected as a potential endosymbiont in mosquitoes (Kikuchi *et al.*, 2005a), *Varroa* mites (Nisbet & Blackwell, 2009), wood boring beetle *(Anoplophora glabripennis*) (Schloss *et al.*, 2006), species of Collembola (Czarnetzki & Tebbe, 2004) and termites, where it has a role in degradation of aromatic compounds and classified as an oxidative/fermentative microorganism (Rani *et al.*, 2009; Zouache *et al.*, 2009a). The role or relationship of *Comamonas* to *P. ovis* was not studied in this thesis as this bacterium was not cultured directly therefore further work is required to understand this association. Survival assays that assess the effect of selectively removing individual endosymbionts would be required to investigate this. Moreover, crossing experiments between infected and non-infected individuals (Konig, 2006) could be carried out to investigate their role further. Before this is possible though, further work is required to maintain and produce favourable reproductive conditions for these mites long term off-host in the laboratory.

Mites were pooled to ensure sufficient sample quantity, however, there were three disadvantages of using pooled whole-crushed mites in this chapter. The first is the lack of prevalence data within the population from individual mites as more than one mite was used for PCRs. Additionally, the presence of *Comamonas* in more widespread geographic populations of *P. ovis* mites is required to determine its suitability for microbial control of sheep scab disease. Furthermore, one mite sample (M3) was positive by PCR for both *Cardinium* and *Comamonas*. Co-occurrence cannot be determined as more than one mite was used and sequence analysis revealed that the

C*ardinium* primers amplified *Comamonas* bacteria. Co-occurrence is fairly rare, and although it is not seen in spiders (Gotoh *et al.*, 2007) it has been detected in mosquitoes (*Aedes albopictus*) (W*olbachia, Comamonas and Pseudomonas)* (Duron *et al.*, 2008) and predatory mites (*Metaseiulus occidentalis)* (W*olbachia* and *Cardinium*) (Zchori-Fein & Perlman, 2004). Secondly, the location of the bacterium within the mite is unknown. Further investigations, using dissected *P. ovis* mites would be required or employing staining/tagging methods (Zouache *et al.*, 2009b). Dissection is particularly difficult as the average size of an adult *P. ovis* is 500 μm. For other host organisms, the particular localisation of the endosymbiont appears to suggest their role within the organism. For example, when located in the ovaries or reproductive tissues the bacterium often has effects on host reproduction. This is seen when *Cardinium* causes feminisation in flat mites (*Brevipalpus* spp) (Seniczak *et al.*, 2009). In spiders, however, *Cardinium* is located elsewhere and affects other life history factors, such as longevity (Duron *et al.*, 2008). Thirdly, as adult mites of both sexes were used for this study, the effect of sex or developmental differences in *Comamonas* spp presence is not known. Some endosymbionts are present in both males and females; for example *Wolbachia* (Van Meer *et al.*, 1999).

It is possible that endosymbiotic bacteria may be present within a host without having any obligate function. W*olbachia* and *Cardinium* have been detected in spiders but there is no evidence that spiders are dependent on either bacteria for survival or reproduction, supported by the lack of fixed infections within a species (Duron *et al.*, 2008). There was no evidence of fixed infections of *Comamonas* in this study and hence their role may not be essential to the mite.

Within *P. ovis*, *Comamonas* spp may act as a secondary (facultative) endosymbiont, which may confer slight positive benefits under particular environmental conditions (Duron *et al.*, 2008), but a cost of the presence of these symbionts has also been observed in a number of organisms (Rocha & Danchin, 2002). A number of enzymes have been idenitifed from *Comamonas* sp (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases) (KEGG, 2011), so this bacterium may contribute to enzymes and/or digestion within the mite.

The lack of other endosymbionts in *P. ovis* in this study is not surprising. Although endosymbionts have diverse host ranges, on an arthropod-wide scale, *Cardinium* has only

been detected in 6% of species screened compared to 24% positive for *Wolbachia* (Kitajima *et al.*, 2007).

The objective of this chapter was to identify bacteria present within *P. ovis* mites and assess their suitability for bacteriophage biocontrol targets. For this, the bacteria need to be culturable to allow the isolation of a specific bacteriophage and also restricted and important to *P. ovis* mites; a bacterium that is part of the normal/essential flora of sheep would not be suitable because of the potential detrimental effects on the sheep. Together with specificity, the function of the bacterium is important. *Pasteurella multocida* and *Erysipelothrix rhusiopathiae*, although both are found in the poultry red mite (*D. gallinae*). are known to have saprophytic or pathogenic effects on the mite (Zchori-Fein *et al.*, 2001; Zchori-Fein & Perlman, 2004). Removal of these bacteria may benefit, rather than harm this parasitic mite. There are no known studies of bacteriophage biocontrol of endosymbionts, athough one bacteriophage against the secondary symbiont of pea aphid, *Acyrthosiphon pisum* has been identified (van der Wilk *et al.*, 1999). This study detected the presence of one potentially-endosymbiotic bacteria from natural and *in vivo P. ovis* mites using a specific PCR assay. *Comamonas* sp, as potentially culturable (Setala, 2000), has the properties to be a target for bacteriophage therapy as a means of controlling *P. ovis* mites.

2.4.2 Assessment of Techniques

2.4.2.1 Microbiological Methods for Bacterial Isolation from Fleece and Mites

This is the first known study of the microbial communities associated with the infected fleece of sheep scab disease. The microbiological technique (biochemical and phenotypical tests) presented evidence for changes in the bacterial community between healthy and scab-infected fleece. There were some limitations in the use of culture-based techniques for bacterial identification as only culturable bacteria were isolated. Therefore this method lacked the diversity of culture-independent bacteria as it is estimated that 99% of environmental bacteria are unculturable in the laboratory (Dillon & Dillon, 2004). The set of microbiological tests used were not able to classify all isolates, requiring further tests to distinguish the unresolved bacteria. This increased workload is a limit to culture-dependent assays. The identification criteria chosen of major groups (Cowan *et al.*, 2003) lacked some taxonomic resolutions, resulting in the

over/under estimation of some bacterial groups. For example, *Pseudomonas* and *Alcaligenes* species were grouped together using the identification criteria. Although both are Proteobacteria, they are in different classes (gamma and beta respectively). This generalist method of classification results in a lack of depth and detail about some of the isolates.

2.4.2.2 *P. ovis* Faecal Trails

P. ovis faecal trails provided a culture-dependent method to isolate bacteria from the internal cavity of the mite. Surface sterilisation, although sometimes fatal to the mites, was critical in this study to ensure removal of bacteria from the external surfaces of the mite. The bacteria isolated may not be representative of the total diversity of bacteria present within *P. ovis* as there may be commensal bacteria enclosed within the internal gut cavity which are not excreted. Faecal trails resulted in the isolation and identification of culturable aerobic bacteria that will subsequently be used as targets for bacteriophage biocontrol in Chapter 4. Culturing of anaerobic bacteria, although potentially illuminating for microbial analysis, would not be suitable targets for bacteriophage biocontrol so were not investigated further in this chapter.

Ten bacteria were isolated and purified from faecal trails. Further sampling would expect to isolate a greater range of bacterial species. Several different agar types and culturing conditions were used to isolate bacteria yet there is still a potential of competition from fast growing organisms over slower growing organisms on the plates (Torsvik *et al.*, 1990). Isolates were initially chosen by their different phenotypic appearances but due to the physical similarity of some species (Eisen 2007), a number of biochemical tests were carried out to differentiate colonies, then identified by DNA molecular sequencing.

2.4.2.3 DNA Extraction

Extracted DNA quantities differed between sample type, with more yielded from fleece than mites. This may be due to the small size of *P. ovis* (mean 500 μm length) (Chapter 1, Figure 1.2A), the initial mass of the sample and hence, the total DNA available to be extracted. The initial mass was affected by the availability of mite samples. Natural samples often had very few mites present so that overall a much smaller number of mites was used for each analysis than previously used by other authors (Pettit *et al.*,

2000; Hogg & Lehane, 2001). Mite samples were pooled for DNA extraction, yet this resulted in a lack of individual mite data as discussed in Section 2.4.1.1. This may be overcome in the future by the use of specialist kits such as Prepman Ultra (Applied Biosystems), which are noted to be efficient for small samples (K. Mounsey *pers comm.*).

The phenol/chloroform method was employed to extract DNA from all the various sample types (mites or fleece) as it was efficient in producing sufficient quantities (and purities) of DNA. A number of alternative methods were evaluated (Henckel *et al.*, 1999), microlysis (K. Stanley, *pers comms*), Proteinase K (Hogg & Lehane, 2001) and the boiling method (Dixon *et al.*, 2007). Greater DNA quantities were extracted from scab-infected compared to healthy fleece, but the reason for this is unclear. Although carried out under similar conditions, individual variation in DNA extraction is expected. The quantity of DNA extracted from mites from the two different origins (*in vivo* and natural), however, was very similar. The extraction method was also robust to degradation that was observed in the natural mite sample. Natural samples were received after a longer off-host period than the *in vivo* culture, all of which were received with 48 h of harvest.

2.4.2.4 PCR methods

Specific group PCR

The molecular techniques used (specific-PCR and ITS PCR) had the same limitations for both mites and fleece samples. One difference was that mite samples were often pooled, whereas each fleece sample was tested individually. To overcome problems with taxonomic classification using microbiological techniques, specific-group PCR was employed to investigate bacterial composition in mites. Specific-group primers were only able to distinguish bacteria at a group level resulting in a lack of detailed identifications. This may lead to over/under estimation of bacterial groups present. The technique of using specific-PCR is time consuming, for both in optimisation and sample screening, as each individual sample had to be screened with each different primer set. This method also lacks detail on the whole bacterial community. For example, *P. ovis* samples only detected Actinobacteria, yet faecal trails had revealed the presence of a number of non-Actinobacteria species.

ITS & RISA for Fleece and Mites

Sequencing of ITS DNA for mite samples was much more successful than for the fleece samples. Yet in contrast, the mite RISA profiles had a lower resolution. The reason for this is unclear. There were a number of initial problems with optimisation of the PCRs for fleece samples. This may be due to organic inhibitors from the fleece environment, such as wool grease (Meyer *et al.*, 2001) interfering with the PCR reactions (Kingsford & Raadsma, 1995). An alternative method for selecting isolates for DNA sequencing would be to cut bands directly from agarose gels. This was advised against for samples in this study due to the low resolution of individual bands on gels (A.Nisbet, *pers comm.*).

Intergenic transcribed spacers (ITS) were also used as primer amplification targets in PCR assays, identifying bacteria by 'phylotypes' (Eisen 2007). These primers gave a 'whole-community' view of the bacterial flora, which was complex for both mite and fleece samples. There were financial constraints which limited the number of phylotypes identified by DNA sequencing.

As a gene target, ITS is extremely good for community analysis using RISA (Cardinale *et al.*, 2004) and for discrimination of closely related organisms (Lee *et al.*, 2009), but has limitations when it comes to sequencing bacterial DNA for identification. This is due to the presence of tRNA genes (ala and ile) and secondary structures within these ITS regions (Figure 2.11) (Iteman *et al.*, 2000). It is also possible to observe artificial bands on agarose gels, caused by heteroduplexes of these structures. These do not occur if PCR products are separated with a denaturing gel (Gurtler & Barrie, 1995; Iteman *et al.*, 2000). Gram negative bacteria contain both $tRNA^{ala}$ and $tRNA^{ile}$ genes, whereas Gram positive bacteria may have only one or both. Additionally some bacteria lack them completely (Gurtler & Stanisich, 1996).

ITS-PCR is more sensitive than using the 16S rRNA gene as it can detect bacterial levels as low as 0.1% of the mixture and can reduce potential PCR bias of preferential amplification of templates in the mixture (Cardinale *et al.*, 2004). 16S rRNA is often believed to be a superior gene target for sequencing due to its long length (for example 1500 bp in *Campylobacter*) and ubiquitous presence in bacteria (Man *et al.*, 2010). In addition, sequence databases such as Genbank have many more 16S than ITS sequences (Danovaro *et al.*, 2006; Man *et al.*, 2010). ITS remains a suitable candidate for bacterial classification as it has been shown to have a higher pairwise percentage difference for

strain differentiation in some bacteria making it the most cost-effective region for differentiation and delineation of systematic relationships (Man *et al.*, 2010).

Another factor that may affect the use of these gene targets are the number of operons found in each bacterium. The *rrn* operon is composed of three genes, 16S rRNA (*rrs*), 23S rRNA (*rrl*) and 5S rRNA (*rrf*), with an ITS region between the 16S and 23S genes. There may be between one and fifteen *rrn* operons per genome, which can in turn affect some molecular techniques, such as RFLP, DGGE and quantitative PCR (Lee *et al.*, 2009). The number of operons, however, can also help with species and strain identification. The number of operons of bacteria found in this study range from one to fourteen (Appendix 8).

Although PCR is extremely efficient at testing presence, primers must be checked carefully to ensure amplification of the desired gene target. Additionally real-time PCR would be required to measure abundance of specific bacterial species identified in this study. This form of PCR utilises fluorescence to quantify DNA present in the sample (Bustin *et al.*, 2009). Moreover, a technique to overcome the limitations of culture and PCR based methods may be the use of metagenomics. This enables the study of genomes of both unculturable organisms and in their natural environment. This technique uses 'shotgun' Sanger sequencing or pyrosequencing for an unbiased sample of all community members. For a detailed review of history and approaches of metagenomics see (Handelsman, 2004; Edwards & Rohwer, 2005; Eisen, 2007).

A further effect of PCR technique may be the cycle number. Studies have shown that phylogenetic diversity of clone libraries decrease with the increase in cycle numbers (Bonnet *et al.*, 2002). Thirty cycles were used in this study for ITS-PCR (Cardinale *et al.*, 2004), therefore the identification and diversity of phylotypes may be increased with reduction in cycle number.

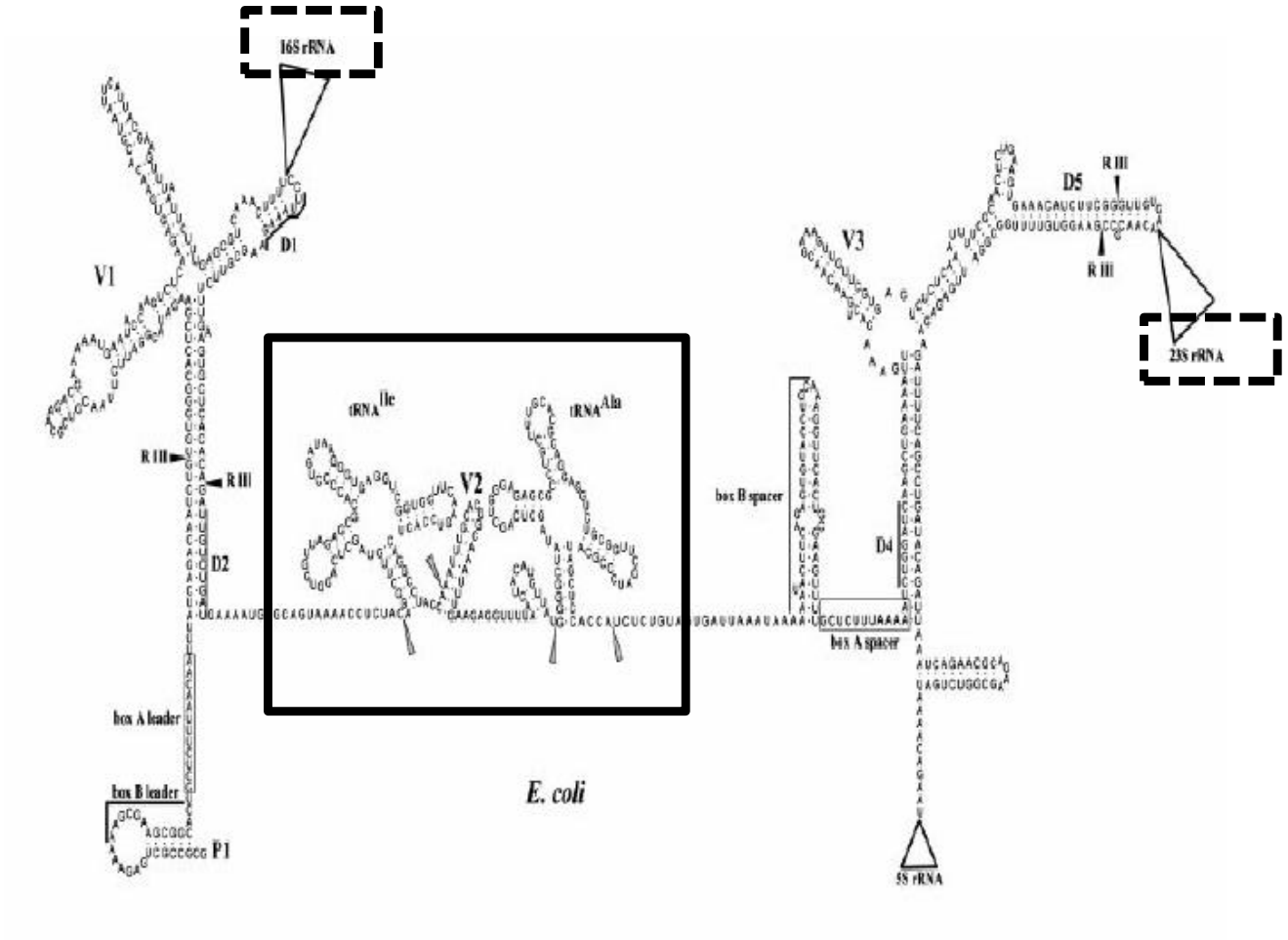

Figure 2.11 Structure of *E.coli rrA* operon from 16S-23S (dashed boxes) showing secondary structures (tRNA^{Ile} & tRNA^{Alu})(solid box). Reproduced from Iteman *et al.,* (2000).

2.4.3 Summary

- Microbial communities associated with sheep scab disease are very complex; No single bacterium appears to be associated with *P. ovis* and scab-infected fleece.
- There were a number of bacterial species (e.g. *Staphylococcus* and *Pseudomonas*) that although present in *P. ovis* are too ubiquitous to be suitable for targeted control of *P. ovis* mites.
- There were a number of bacteria only identified from *P. ovis* in this study (e.g. *Corynebacterium* and *Propionibacterium acnes*).
- One promising microbial target (at this stage of the study) is *Comamonas* sp. This is a known arthropod symbiont (Zouache *et al.*, 2009b) that if disrupted may interrupt the lifecycle of *P. ovis* mites.

3 *In vitro* Survival Experiments

3.1 Introduction

Bacteria associated with *Psoroptes ovis* mites were identified in Chapter 2. This chapter will investigate whether these bacteria could be disrupted through the use of antibiotics and what, if any, the consequences, of disrupting these associated bacterial communities might be on the survival of *P. ovis*.

3.1.1 Maintaining *P. ovis* Mites in the Laboratory

Since *P. ovis* mites spend their entire lifecycle on sheep, *in vivo* (on-host) cultures are essential for understanding the full dynamics of sheep scab disease. Suitable *in vitro* (off-host) maintenance is therefore required to enable research into these mites without the use of live sheep for culturing.

Under natural conditions, *P. ovis* has been observed to live and remain infective off-host for up to 16 days but attempts at culturing *P. ovis* in the laboratory have had varying results (Table 3.1). The longest survival recorded off-host for *P. ovis* was 17 days at 1-8°C, where mites were kept with wool in a nylon stocking (O'Brien *et al.*, 1994a), and 24 days at 15°C with 99% relative humidity for *P. cuniculi,* a cosmopolitan species of *P. ovis* (Arlian *et al.*, 1981). Mathieson (1995) investigated a number of *in vitro* designs, similar to the glass chambers constructed by Smith *et al.,* (1999) which maximised relative humidity as *P. ovis* are free-living they must maintain a water-balance in a range of environments. In addition to survival, the conditions required for successful reproduction of *P. ovis* are difficult to replicate *in vitro* (Mathieson, 1995).

This chapter focuses on the survival of *P. ovis* under *in vitro* conditions, in response to disruption of their internal bacterial communities. The timescales required for *in vitro* survival experiments are short, with significant effects often discernible after a few days. For example, Wall & Bates (2011) tested *trans*-cinnamic acid ethylester for its fatal properties towards *P. ovis* and significant mortalities were observed within 24 h.

The exact composition of *P. ovis*' diet on sheep is unknown (Mathieson, 1995; Beetham, 1997), although it is thought to include serous exudates which arise from allergic dermatitis of the scab infection (Mathieson, 1995). *P. ovis in vitro* dietary preferences have been highlighted to include water, plasma, serum and low-salt diets (Sinclair & Filan, 1989). Moreover, the ingested diet of the mite may change over the course of an infection, as the relatively uncontaminated fleece is affected by ruptured dermal vesicles

and bacteria (Sinclair & Filan, 1989), which may in turn contribute to the mites' diet. A number of bacterial species have previously been identified on healthy and scab-infected fleece, including *Bacillus* spp., *Staphylococcal* spp. and *Pseudomonas* spp. (see Chapter 2).

Table 3.1 Summary of laboratory mite longevity studies of *P. ovis*. Mean Survival for *P. ovis* (except Arlian *et al* 1981: *P. cuniculi*). *Environmental denotes survival of *P. ovis* in the environment (off-host).

Mean survival (days)	Temperature (°C)	Relative humidity (%)	Reference
12	Environmental*	-	Wilson *et al* 1977
15		-	Sargison *et al* 1995
16		-	Thind and Ford 2003
48		-	Liebisch et al 1985
14-17	1ºC- 8ºC	-	O'Brien *et al* 1994b
6	32ºC	95%	Mathieson 1995
20-24	10-20ºC	99%	Arlian *et al* 1981
(5)-13	9ºC - 30°C	95%	Smith et al 1999

3.1.2 Determination of Feeding of *P. ovis In Vitro*

P. ovis mites cultured on-host have been shown to feed on sheep-derived compounds. Pettit *et al*., (2000) used 0.5 ml of packed, clean mites within 2 h of harvesting with an enzyme-linked-immunosorbent assay (ELISA) that could detect ovine antibody immunoglobulin G (IgG) between 10 and 250 ng/ml. They observed sheep IgG concentrations of 192 mg/ml in the mite homogenate, indicating that *P. ovis* ingests host immunoglobulin when feeding.

The separation of rabbit IgG specifically from whole-mite samples can be achieved with the use of antibodies. Once isolated, IgG can be visualised and quantified by a number of methods; such as immunodiffusion with radiolabelled immunoglobulins (Chinzei & Minoura, 1987; Benyakir, 1989), precipitin test (Weitz, 1956), ELISA (Benyakir, 1989; Blackwell *et al*., 1994; Clausen *et al*., 1998), sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (Mathieson, 1995) and PCR (Imura *et al*., 2010). Sandwich ELISA, where the target antigen is 'sandwiched' between two different antibodies, is an especially suitable technique as it is quantitative, very specific and uses fluorescence to detect presence of antigens.

3.1.3 Methods to Disrupt Bacteria

A diverse range of bacteria have been detected in *P. ovis* mites (Chapter 2) yet although the importance of these organisms to the survival of the mite is not known, Hogg & Lehane (2001) noted many species identified were characterised by their ability to produce extracellular lipase, which may aid digestion within the mite.

To investigate the roles of associated bacteria, hosts are commonly treated with either heat-treatment (van Opinjen & Breeuwer, 1999), lysozyme injection, which destroys symbiont cell membranes (Nogge, 1981) or the most common, antibiotic-treatment.

Antibiotics may be administered to hosts through a number of routes; diets are frequently supplemented with them (Wilkinson, 1998; Yusuf & Turner, 2004; Douglas *et al.*, 2006; Son *et al.*, 2008; Lehman *et al.*, 2009); injection (Nogge, 1981; Koga *et al.*, 2007) and aerosols (Ando *et al.*, 1971) have also been used. Direct inhibition of feeding due to antibiotics, however, has not been observed (Ben-Yosef *et al.*, 2008). A variety of effects of antibiotics have been reported on survival (Koga *et al.*, 2007), fecundity (Zhong *et al.*, 2007; Son *et al.*, 2008) and growth (Bandi *et al.*, 1999; Hardie & Leckstein, 2007), through the disruption of internal bacterial communities (See Section 1.3.1). Removing internal bacteria from the host is not always detrimental; the selective removal of the bacterium *Serratia marcescens* from *P. cuniculi* did not prevent the mite from causing clinical problems in rabbits (Perrucci *et al.*, 2005).

As *in vitro* maintenance of *P. ovis* mites is not possible, it is not yet possible to investigate the effects of antibiotics on reproduction or development of the mites.

3.1.4 Action of Antibiotics

Antibiotics have different modes of antimicrobial action but are classed as either bactericidal (bacteria killing) or bacteriostatic (limiting growth). Some are broad spectrum, acting against both Gram positive and negative bacteria, whereas others are more specific with different modes of action (Hahn & Sarre, 1969) (Table 3.2). There are a number of techniques to test antimicrobial susceptibility including the zone of inhibition test (ZOI) (aka Kirby-Bauer method) (Sutter & Finegold, 1971; Boyle *et al.*, 1973).

Table 3.2 Bacterial targets and mode of action of five different antibiotics.

Antibiotic	Bacterial Targets	Mode of Action
Gentamicin	Gram negative	Aminoglycosides
Tetracycline	Gram positive & negative	Inhibition of protein synthesis
Ampicillin	Gram positive	β- lactamase
Penicillin	Gram positive & negative	Inhibition of bacterial cell wall synthesis
Chloramphenicol	Gram positive & negative	Inhibition of protein synthesis

3.1.5 Aims

The work described in this chapter aimed to investigate the effect of disrupting the bacterial communities of *P. ovis* mites by the administration of antibiotics and assessing the effect through survival and bacterial density of *P. ovis* and bacteria associated with these mites.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Chemicals and Media

All antibiotics and chemicals were sourced from Sigma (Sigma Ltd., UK). Microbiological media; nutrient agar (NA; CM0003), nutrient broth (NB; B00210) and maximum recovery diluent (MRD; CM0733), were sourced from Oxoid, UK and made up according to manufacturers' instructions.

3.2.1.2 *P. ovis* Mites

Mite samples (mix of males and females) were received from a *in vivo* culture at The Moredun Research Institute, Edinburgh. Samples were received 24-to-48 h following harvest from sheep (Section 2.2.1.3).

3.2.1.3 Mite Faecal Bacteria

Mite faecal bacteria (MFB) used for antibiotic susceptibility tests (Section 3.3.3) were originally isolated from *P. ovis* faecal trails (Section 2.3.2, Table 2.7).

3.2.2 Methods

3.2.2.1 Maintenance of Mite Faecal Bacteria

Mite faecal bacteria (MFB) were maintained as previously described (Section 2.2.2.5).

3.2.2.2 Maintenance of *P. ovis* Mites in the Laboratory

To maintain live mites in the laboratory, chambers were constructed based on Mathieson (1995), using two Perspex slides (30 mm x 60 mm) with a 15 mm diameter hole cut in the centre of both. A 25 mm sterile circular filter paper disc (Grade 1 Whatman) was placed in between the slides and secured with elastic bands or masking tape at each end (Figure 3.1). Mites were then placed, using either a mounted needle modified with a plastic hair or a fine paint brush, onto the filter paper and a cover slip secured on top with two elastic bands.

Water and lamb serum (Invitrogen), two previously reported preferred diets of *P. ovis* (Sinclair & Filan, 1989) were tested initially, by administering 10 µl to the underside of the filter paper, daily. Mite chambers were then placed onto a moist tray of cotton wool and covered loosely with cling film to maintain a high relative humidity (Mathieson, 1995; Smith *et al.*, 1999). The chambers were placed in an incubator at 24 ± 2°C and

humidity was measured using a HT50 meter (A.T.P). The positions of the chambers on the tray were rotated daily to avoid any incubator effects. Mite chambers were checked daily under a dissecting microscope to ensure sufficient moisture levels in the chambers and also for mortality; mites were pronounced dead if they failed to move in response to manipulation with a paintbrush.

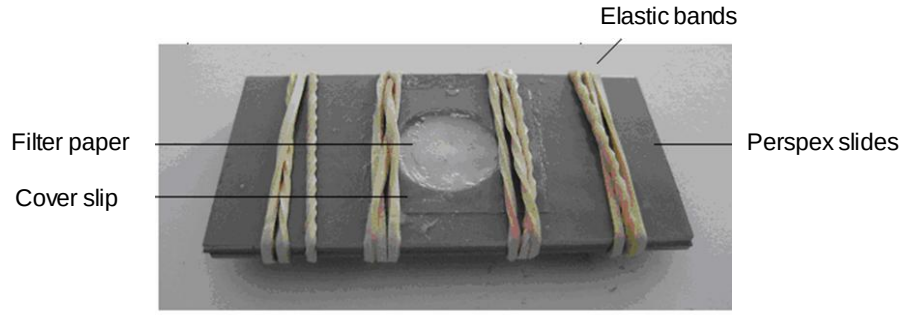

Figure 3.1 Laboratory *in vitro* chamber. *P. ovis* mites were placed in the centre hole between the filter paper and cover slip.

3.2.2.3 Determination of *P. ovis* Feeding

Enzyme-linked immunosorbent assays (ELISA) were carried out to confirm that *P. ovis* mites were feeding in the experimental laboratory chamber setup (Section 3.2.2.2).

Feeding Assays for ELISA

Mites were setup in chambers as previously described (Section 3.2.2.2) and fed either a negative control (water), host serum (lamb) or non-host serum (rabbit). A sub-sample of mites was directly frozen at -20°C after receipt (Section 3.2.1.2) as an *in vivo* host-fed positive control. Mites were fed and checked daily for mortality. A random sample of mites were removed daily from each treatment and immediately frozen at –20°C, for five days.

Preparation of Mite Samples for ELISA

After the feeding assay, frozen mites were washed repeatedly in SDS (2%) to remove any host material from the external surface (Pettit *et al.*, 2000). Then one mite was transferred to a tube, to which 100 µl of carbonate-bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6), was added. Carbonate-bicarbonate buffer was prepared freshly for each experiment and stored at 4°C (Blackwell *et al.*,

1994). The tubes were sonicated twice, for 3 min, with a Transsonic T310 (Camlab). To ensure that mites were fully macerated, a sterile plastic pestle was then used to disrupt the cells. These whole-mite extracts were used in the ELISA.

ELISA Kit

A SensiFlex™ ELISA development kit for mouse IgG with Fluorocillin™ Green (Invitrogen) was used following the manufacturer's instructions in a sandwich ELISA. The capture antibody used was goat anti-rabbit general IgG (Sigma; 10 μg/ml) and the detection antibody was mouse anti-rabbit IgG (Sigma; 10 μg/ml), both diluted in 50 mM $NaHCO_3$ buffer. B-lactamase conjugate (10 μg/ml) antibody binds to the detection antibody and cleaves the substrate, Fluorocillin Green Reagent (0.9 μM) to produce a fluorescent signal. B-lactamase activity was measured with an Omega Fluorostar plate reader with 495 nm excitation and 525 nm emission. The antigen standards used were neat rabbit serum diluted by twelve ten-fold serial dilutions in 0.1X PBS-BSA. Each ELISA well had 100 μl of sample or antigen standard, in triplicate. A spectrophotometer (Nanodrop ND-1000) was used to measure the quantity of IgG present in the antigen dilutions.

3.2.2.4 Assessment of Antibiotics for *In Vitro* Feeding Assays

Antibiotics

Antibiotic dilutions (Table 3.2) were made with sterile distilled water except for tetracycline which was initially dissolved in 0.1 M HCl then in distilled water and chloramphenicol, which was dissolved in ethanol followed by distilled water. All antibiotics were made up on the first day of the experiment, aliquoted and frozen at -20°C until use.

Zone of Inhibition Assay

Zone of inhibition (ZOI) of bacteria in response to antibiotics was measured with the aim of producing a reference to quantify the amount of antibiotic ingested by mites in the feeding experiments (Section 3.2.2.5). Double agar plates were set up for each MFB (Adams, 1959) by adding 100 μl of overnight MFB liquid culture (Section 2.2.2.5) to a sterile tube containing 3.5 ml of molten top agar (NB + 0.06% agar) cooled to 47°C, mixed gently then poured on top of a hardened NA plate. The top agar was allowed to set then to each plate, six antibiotic assay discs (5 mm, Whatman) were evenly distributed around the agar with flame-sterilised tweezers. To a single disc, 5 μl of

antibiotic in one of five concentrations (5, 25, 50, 100 and 200 μg/ml) was added. Different antibiotics were tested on separate plates (gentamicin, tetracycline, ampicillin, penicillin and chloramphenicol), setup in duplicate. In addition a diluent-only (Section 3.2.2.4) negative control disc was included. Plates were incubated at 27°C for 24 h and then the zone of inhibition (ZOI) (Brock, 1970) was measured using a ruler (Figure 3.2).

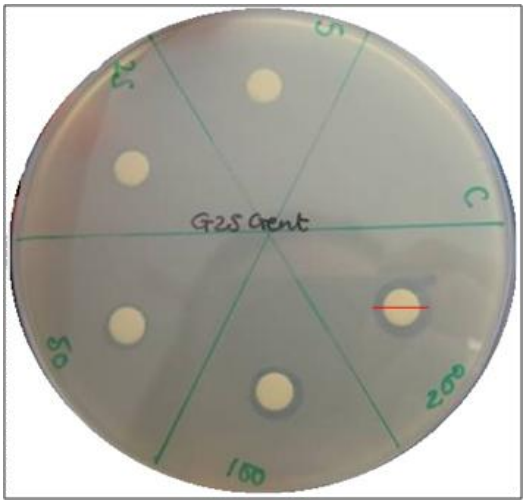

Figure 3.2 Measurement of zone of inhibition (ZOI) of antibiotic on an agar plate shown by red line.

Antibiotic Effect on Liquid Mite Faecal Bacterial Cultures

The affect of antibiotics on MFB in liquid phase was investigated to determine the suitability of optical density (600 nm) as a measure of bacterial density in addition to investigation of antibiotic effect when bacteria are in a different phase.

Following the ZOI assay results, gentamicin and tetracycline were the most effective antibiotics against MFB. Overnight MFB liquid culture (100 μl) (approximately Log7 CFU) was inoculated into 10 ml NB. To this, antibiotic of different concentrations was added (6.25, 12.5, 25, 50, 100, 200, 500 μg/ml), setup in duplicate. Controls of NB only, bacteria only and NB with antibiotics were also setup. Cultures were incubated at 27°C on an orbital shaker and optical density (OD) (600 nm) (Eppendorf Biophotometer) was measured periodically over 48 h. At the same time as OD measurement, 1 ml of culture was removed, diluted in maximum recovery diluent (MRD) and plated on NA plates. Plates were incubated overnight at 27°C and colony forming units (CFU/ml) were calculated.

3.2.2.5 Effect of Antibiotics on Survival of *P. ovis* mites

Microinjection of Antibiotics into *P. ovis* Mites

Microinjection of antibiotics was investigated to ensure antibiotics were delivered directly into the mite gut, where bacteria are thought to reside.

A Transjector 5246 (Eppendorf) with 0.5 μm femtotips (Eppendorf) was used at a constant pressure of 80 psi for a range of injection times (0.1, 0.5, 1 and 5 s). The average volume of an adult *P. ovis* mite was calculated based on a scalene ellipsoid (Equation 3.1) using *P. ovis* measurements from SEM images. The aim was to inject no more than 1% of the total mite volume (Jasinskiene *et al.*, 2007).

Mites were cooled on ice and then stuck upside down onto double sided tape in a line (Figure 3.3) (Presnail & Hoy, 1992) on top of four microscope slides and the needle was inserted at a 150° angle, moving only the microscope stage. A back pressure of about 100 psi (Terenius *et al.*, 2007) was used. The needle was aimed at the centre on the side of the gut cavity to minimise damage (Mathieson, 1995). In addition a needle-stick-only and non injection control was tested. Mortality was checked every 2 h for the first 8 h.

Equation 3.1 Equation to estimate volume of a single *P. ovis* mite. a = radius along x axis, b = radius along y axis, c = radius along z axis.

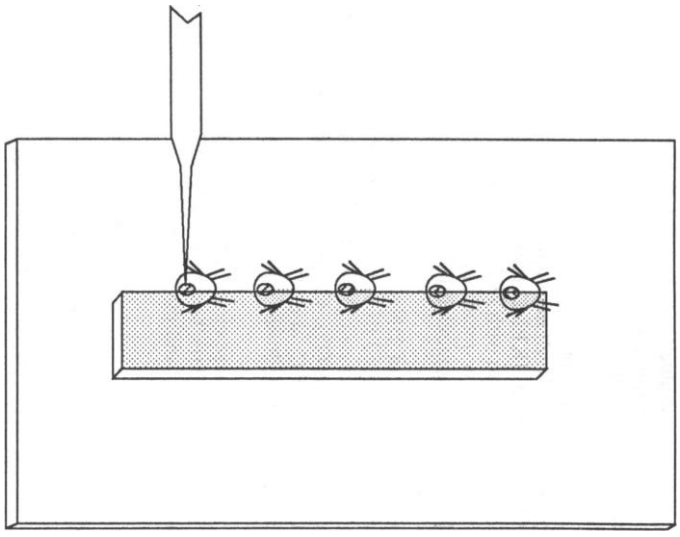

Figure 3.3 Diagram of microinjection of mites (Presnail & Hoy 1992).

In vitro Feeding Experiments Assessing the Effect of Antibiotics on *P. ovis* Survival

To investigate the effect of disrupting bacterial communities within mites, antibiotics were administered to mites in food.

Adult mites of mixed sexes received from an *in vivo* culture within 48 h of harvest from sheep (Section 3.2.1.2) were randomly allocated to mite chambers as previously described (Section 3.2.2.2). Five replicates of each treatment were used for the first three experiments (Section 3.3.4.1-3.3.4.3) and nine replicates for the pilot bacterial density experiment (Section 3.3.4.4) with a maximum of 20 mites per chamber. Mites were fed 10 µl of each treatment diet (antibiotics, lamb serum or water) daily by administration using a pipette onto the underside of the filter paper. Three antibiotics were tested (ampicillin, gentamicin or tetracycline) at two different concentrations (50 µg/ml, 100 µg/ml) dissolved either in water or lamb serum (to encourage feeding and ingestion of the antibiotics). In each experiment lamb serum was used as the preferred serum control diet and water was used as a 'no food' control. Survival of mites was recorded daily. Any dead mites were removed from the chambers and frozen at -20°C for subsequent analysis. The chambers were all kept on a plastic tray with damp cotton wool (to produce a humid environment) in a 24-28°C incubator. The positions of the chambers on the tray were rotated daily to avoid any incubator affects.

Effect of Antibiotics on *P. ovis* Bacterial Density

Following experiments on survival (Section 3.2.2.5), antibiotics (100 µg/ml gentamicin and tetracycline) were administered to mites to observe effects on internal bacterial density. The pilot experiment (Section 3.3.4.3) had 20 mites per chamber with three replicates of each treatment setup, whereas the bacterial density experiment (Section 3.3.4.4) had 20 mites per chamber, with nine replicates of each treatment.

Mites (alive and dead) were removed from the chambers daily (in the pilot experiment; Section 3.3.4.3), or at 12 h intervals (Section 3.3.4.4) to measure internal bacterial density. The mites were individually surface sterilised as previously described (Section 2.2.2.5) then macerated with sterile tweezers and mixed with 100 µl of ¼ strength Ringers solution (supplemented with 0.2% peptone). A ten-fold dilution series in Ringers solutions was prepared from this whole-mite extract and 100 µl of three appropriate dilutions were plated onto NA. The plates were incubated at 27°C for 24 h before CFUs were counted.

Antibiotic Intake by Mites

To measure the antibiotic intake by mites during *in vitro* feeding assays the ZOI and spot lysis methods were used as previously described (Section 3.2.2.4). To do this, 5 µl of whole-mite extract was spotted onto previously MFB-inoculated plates. Plates were incubated at 27°C for 24 h and the ZOI area was measured to establish whether mite carcasses contained any ingested antibiotic.

3.2.2.6 Statistics

ELISA analysis was performed in Omega Data analysis (Omega). Effect of antibiotics on liquid MFB (Log_{10} Mean CFU/ml) was analysed with Kruskal-Wallis in Minitab (v15) (MiniTab Inc). All survival analyses (Log rank equality of curves and General linear mixed model (GLMM)) were carried out in Genstat (v11.1) (VSN International Ltd, UK) except Probit analysis (LT_{50}) which was calculated in MiniTab. Bacterial density values (CFU/mite) were transformed (Log_{10}+1) then analysed using a Linear mixed model (LMM) and regression equations in Genstat (v11.1) .

3.3 Results

3.3.1 Maintenance of *P. ovis* Mites in the Laboratory

Preliminary experiments with different diets produced a range of survival times, (between 2 and 12 d) with the maximum observed with lamb serum. The mean survival time was 8 d for mites fed with lamb serum (data not shown).These preliminary tests also optimised parameters such as frequency of filter paper changes, to every 2 d. One key parameter that was not possible to measure was the relative humidity inside individual mite chambers, due to the size of the chambers. Moisture levels in the chambers were therefore determined by inspection of individual chambers. Mites used for these tests were from an *in vivo* culture which were received fully engorged within 48 h of harvest. Mites from natural infections were not used due to the uncertainty of time the mites had been off-host.

3.3.2 Determination of *P. ovis* Feeding

3.3.2.1 Using ELISA

Investigating Rabbit Serum as a Novel Control Diet

Rabbit serum was tested as an alternative *in vitro* food to lamb serum to distinguish from on-host feeding and to determine whether *P. ovis* were feeding in the *in vitro* chambers. There was no significant difference observed in survival time between mites fed either lamb or rabbit serum as compared by LT_{50} values from these treatments fed lamb (3.59 d) or rabbit serum (3.76 d) or water (2.97 d). (Table 3.3; Figure 3.4). Therefore rabbit serum was concluded to be suitable for use in the ELISA-feeding experiment as a control diet to distinguish *in vitro* chamber feeding from previous on-host feeding.

Table 3.3 LT_{50} values for *P. ovis* mites fed rabbit serum, lamb serum or water. Values followed by the same letters are not significantly different

Treatment	Mites alive at start (0 d)	Mites alive at end (6 d)	LT_{50}	Standard Error	95% Fiducial CI	
					Lower	Upper
Lamb serum	30	0	3.58a	0.19	3.21	3.98
Rabbit serum	30	0	3.77a	0.23	3.33	4.24
Water	30	0	2.97a	0.18	2.61	3.33

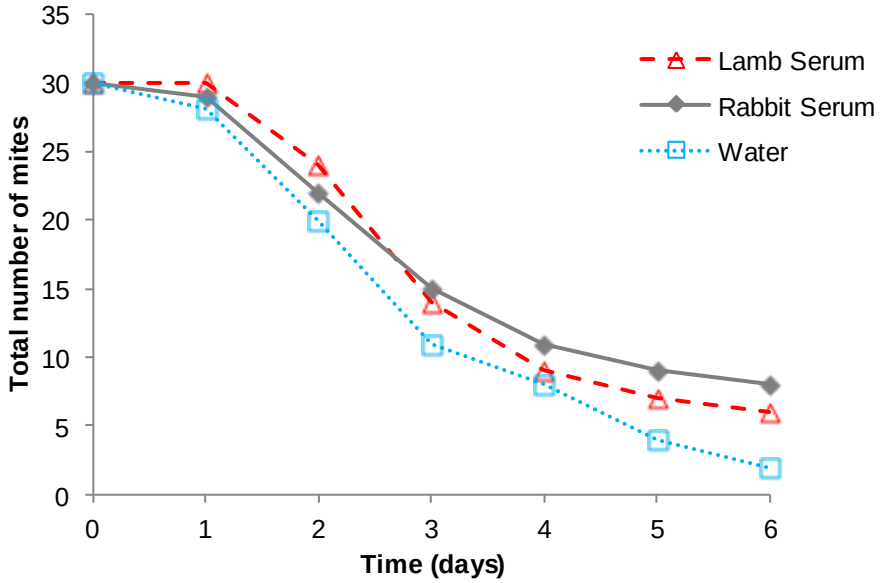

Figure 3.4 Survival curves for *P. ovis* mites (with LT_{50} values) fed lamb (3.59 d) or rabbit serum (3.76 d) or water (2.97 d). Number of mites at start = 30 per treatment.

Optimisation of Rabbit IgG ELISA

The ELISA kit to detect rabbit serum in *P. ovis* mites was first optimised using neat rabbit and lamb serum to produce linear regression fit (R^2) of 0.67, indicating a good correlation between fluorescence and IgG concentration (mg/ml). In addition, water and lamb serum controls were not detected with this kit, therefore the setup was determined to be specific to rabbit IgG and not ovine IgG, making it suitable for determination of mite feeding of rabbit serum in the laboratory chambers.

ELISA Feeding Experiment and Detection of Rabbit IgG

P. ovis mites were set up in *in vitro* chambers, fed rabbit serum, lamb serum or water and five mites per time point (daily for 5 d) were removed and frozen at -80°C and survival of remaining mites was measured (Figure 3.5). There was no difference in survival time of *P. ovis*, with LT_{50} values of 2.89 d±0.09, 2.83 d±0.10 and 2.53 d±0.09 for rabbit, lamb and water respectively, indicating no significant difference between treatments (Table 3.4).

Frozen mites were then prepared for ELISA and aliquoted into three replicate wells of the ELISA plate. The standard curve produced (R^2 = 0.68) was comparable to the optimised control plate. Rabbit IgG was not detected from mites fed either lamb serum or water, however, rabbit IgG was detected from *P. ovis* mites fed rabbit serum in the *in vitro* chambers (Figure 3.6). In addition *P. ovis* mites directly from harvest (i.e. not fed in the laboratory) were also tested and no rabbit IgGs were detected.

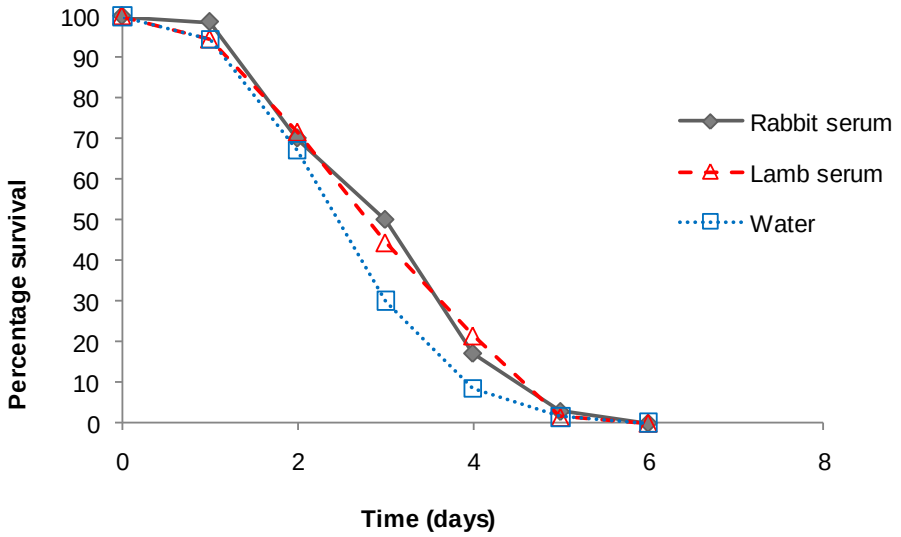

Figure 3.5 Survival curves for *P. ovis* mites fed lamb or rabbit serum. Number of mites at start = 70 per treatment. LT_{50} values: Lamb serum (2.83 d), Rabbit serum (2.89 d), water (2.53 d).

Table 3.4 LT_{50} values calculated by probit analysis to determine the suitability of rabbit serum as an alternative to lamb serum or water. Values followed by the same letter are not significantly different.

Treatment	Mites alive at start (0 d)	Mites alive at end (6 d)	LT_{50}	Standard Error	95% Fiducial CI	
					Lower	Upper
Lamb serum	70	0	2.83a	0.10	2.64	3.02
Rabbit serum	70	0	2.89a	0.09	2.71	3.08
Water	70	0	2.53a	0.09	2.35	2.71

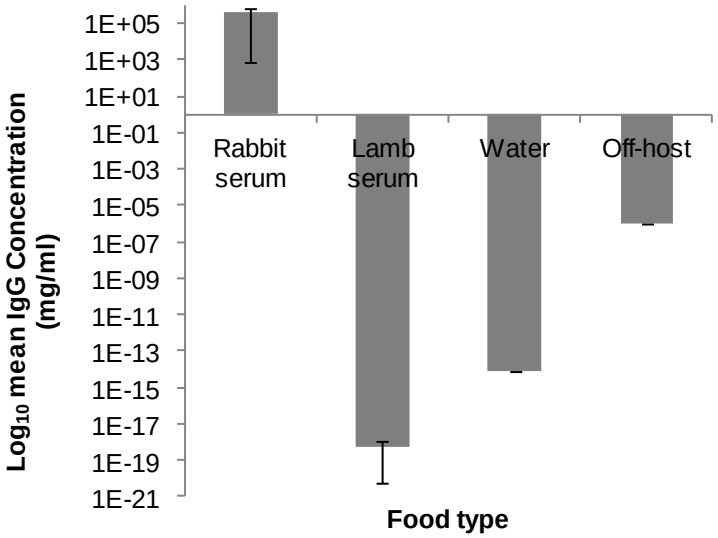

Figure 3.6 Histogram of Log_{10} mean IgG concentration (mg/ml) detected from mites. Mites were fed rabbit serum, lamb serum or water in *in vitro* feeding chambers. Also 'off-host' were mites tested directly after harvest from sheep. Error bars are sem. Only rabbit IgG was detected with this sandwich ELISA.

3.3.3 Determination of Suitable Antibiotics for *In Vitro* Mite Feeding Experiments

To investigate the effect of disrupting internal bacterial communities associated with *P. ovis,* five different antibiotics were tested for their effects on growth of the ten mite faecal bacteria (MFB).

3.3.3.1 Zone of Inhibition Assay using Antibiotics on MFB

Zone of inhibition (ZOI) assays provide a rapid method to test the efficacies of antibiotics on bacterial samples by producing an area devoid of bacterial growth on an agar plate. A range of ZOIs were observed for five antibiotics against MFB, the magnitudes of which are standardised for each antibiotic and therefore can assist in classifying whether bacteria are moderately susceptible (MS) or susceptible (S) to the antibiotic (Table 3.5) (Larkin, 1975). All bacteria were resistant to ampicillin based on ZOI, whereas tetracycline, gentamicin and penicillin had inhibitive effects on growth of all MFB.

Table 3.5 Zone of inhibition (ZOI) results for mite faecal bacteria (G16-G27) with five antibiotics (gentamicin, tetracycline, ampicillin, penicillin and chloramphenicol). Bacteria were classed as moderately susceptible (MS; highlighted in green) and susceptible (S, highlighted in red) by ZOI values (Larkin, 1975). Numbers indicate the mean zone of inhibition in mm (N=2). Blanks indicate no inhibition of growth seen (bacteria resistant to antibiotic).

Mite Faecal Bacteria	Bacterial species	Gentamicin (µg/ml) MS>12, S>15						Tetracycline (µg/ml), MS>14, S>18						Ampicillin (µg/ml), MS<10, S>14						Penicillin (µg/ml), MS<13, S<17						Chloroamphenicol (µg/ml), MS>13, S>18					
		0	5	25	50	100	200	0	5	25	50	100	200	0	5	25	50	100	200	0	5	25	50	100	200	0	5	25	50	100	200
G16	*Staphylococcus aureus*		7	10	11	12	12		8	12.5	16	19	22									15	20	23	25					7	16
G17	*Carnobacterium mobile*																														
G18	*unidentified*			8	8	9	12.5			11	12	18	20							6.5	15	21	27	30					7	9	16
G19	*Micrococcus luteus*				13	15	20			9	12	12	15																	8	10
G22	*Uncultured bacterium O1_44*									6.5	9	11	14																		
G23	*Alcaligenes faecalis*				7	7	7					6.5	8		7	7	8	8	8			7	7	7	7						
G24	*Micrococcus luteus*		7	7	9	10	13			8	8	9	19																		
G25	*Escherichia coli*			6.5	7	9	11				8	11	15				7	6.5	6.5		7	10	13	17	22					7.5	10
G26	*Bacillus cereus*		7	8	9	10	11				16	18	19															7	7	8	13
G27	*Uncultured bacterium O1_44*												7																		

3.3.3.2 Gentamicin and Tetracycline Effect on Liquid Mite Faecal Bacterial Cultures

Zone of inhibition (ZOI) assays demonstrated the effect of different antibiotics on MFB. The tests revealed that gentamicin and tetracycline were effective antimicrobials against MFB in solid states so their effects on MFB growth in liquid were investigated. Three MFB were chosen (*S. aureus* G16; *A. faecalis* G23; *E. coli* G25) as bacteriophage infective against these had been isolated (Chapter 4). Growth was measured by OD and then converted to CFU/ml using standard curves specific to each MFB (Appendix 9).

Effects of Tetracycline on MFB in Liquid Culture

Effect on *S. aureus*

All concentrations of tetracycline (50, 100, 200, 500 μg/ml) inhibited the growth of *S. aureus* (G16) considerably in liquid (Figure 3.7 Ai). There was a significant effect of antibiotic treatment on *S. aureus* growth (H_5=16.83, P=0.005) with Log_{10} CFU/ml medians of *S. aureus* only (7.446), with 50 μg/ml tetracycline (7.121), with 100 μg/ml tetracycline (7.111), with 200 μg/ml tetracycline (7.116), with 500 μg/ml tetracycline (7.130) and for LB only (7.058). There did not appear to be any dose-dependent response to this antibiotic (Figure 3.7 Aii).

Effect on *A. faecalis*

All concentrations of tetracycline (50, 100, 200, 500 μg/ml) inhibited the growth of *A. faecalis* (G23) considerably in liquid (Figure 3.7 Bi). There was a significant effect of antibiotic treatment on *A. faecalis* growth (H_5=23.3, P<0.001) with Log_{10} CFU/ml medians of *A. faecalis* only (7.377), with 50 μg/ml tetracycline (6.918), with 100 μg/ml tetracycline (6.939), with 200 μg/ml tetracycline (6.950), with 500 μg/ml tetracycline (6.966) and for LB only (6.898). There did not appear to be any dose-dependent response to this antibiotic (Figure 3.7 Bii).

Effect on *E. coli*

All concentrations of tetracycline (50, 100, 200, 500 μg/ml) inhibited the growth of *E. coli* (G25) considerably in liquid (Figure 3.7 Ci). There was a significant effect of antibiotic treatment on *E. coli* growth (H_5=23.77, P<0.001 with Log_{10} CFU/ml medians of *E. coli* only (7.928), with 50 μg/ml tetracycline (7.294), with 100 μg/ml tetracycline (7.303), with 200 μg/ml tetracycline (7.315), with 500 μg/ml tetracycline (7.319) and for

LB only (7.267). There did not appear to be any dose-dependent response to this antibiotic (Figure 3.7 Cii).

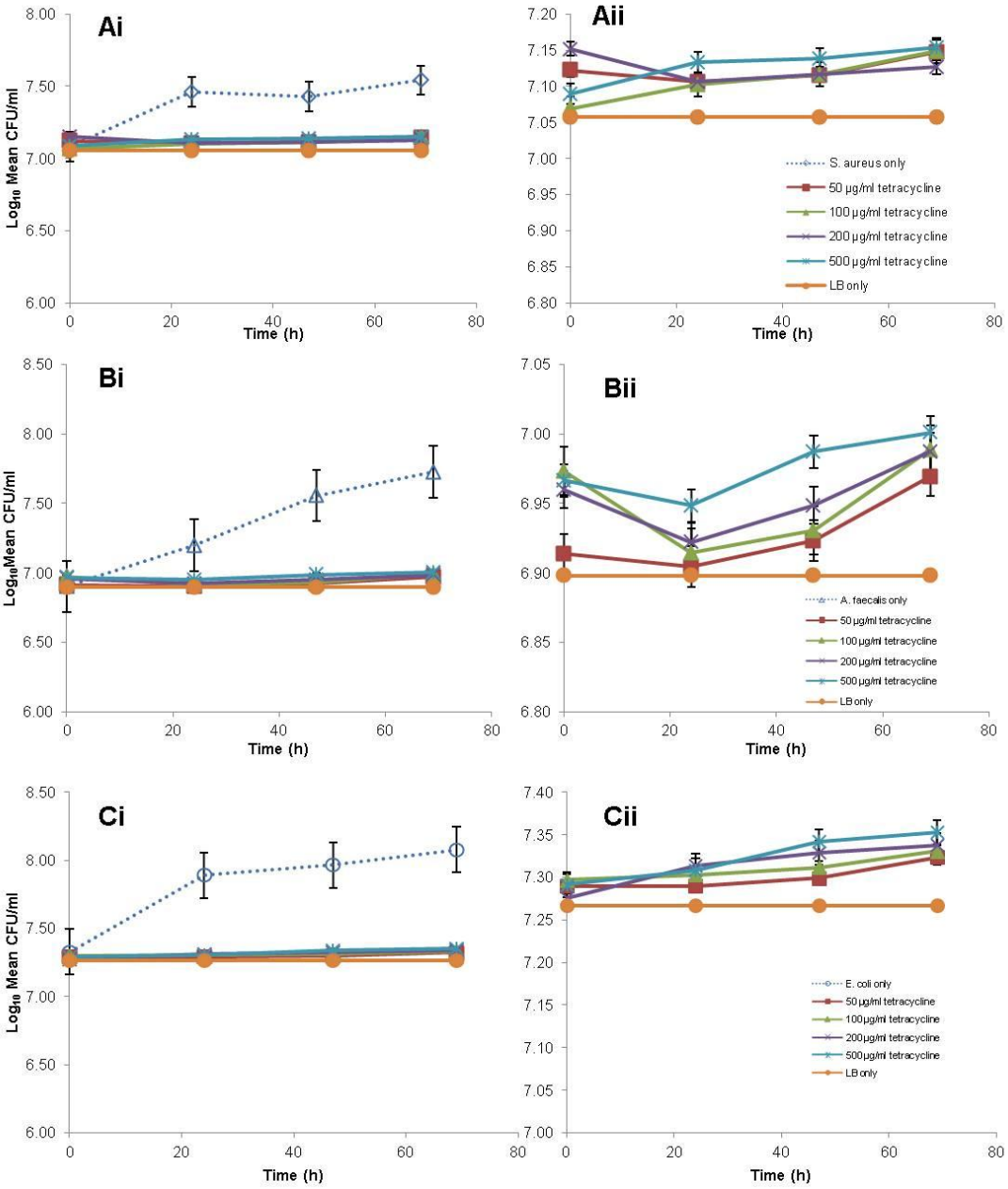

Figure 3.7 Effect of tetracycline (50, 100, 200 and 500 µg/ml) on MFB in liquid culture. *S. aureus* (G16; **A**), *A. faecalis* (G23; **B**) and *E coli* (G25; **C**), with (**i**) and without (**ii**) bacteria controls. Error bars are ±1 standard error (s.e). N=3 for each treatment and time point.

Effects of Gentamicin on MFB in Liquid Culture

Effect on *S. aureus*

All concentrations of gentamicin (6.25, 12.5, 25 μg/ml) inhibited the growth of *S. aureus* (G16) considerably in liquid (Figure 3.8 Ai). There was a significant effect of antibiotic treatment on *S. aureus* growth (H_4=19.08, P=0.001) with Log_{10} CFU/ml medians of *S. aureus* only (7.846), with 6.25 μg/ml gentamicin (7.093), with 12.5 μg/ml gentamicin (7.086), with 25 μg/ml gentamicin (7.090) and for LB only (7.058). There did not appear to be any dose-dependent response to this antibiotic (Figure 3.8 Aii).

Effect on *A. faecalis*

All concentrations of gentamicin (6.25, 12.5, 25 μg/ml) inhibited the growth of *A. faecalis* (G23) considerably in liquid (Figure 3.8 Bi). There was a significant effect of antibiotic treatment on *A. faecalis* growth (H_4=25.6, P<0.001) with Log_{10} CFU/ml medians of *A. faecalis* only (7.675), with 6.25 μg/ml gentamicin (6.899), with 12.5 μg/ml gentamicin (6.898), with 25 μg/ml gentamicin (6.899) and for LB only (6.898). There did not appear to be any dose-dependent response to this antibiotic, except for a rise in growth at 72 h with 6.25 μg/ml gentamicin (Figure 3.8 Bii).

Effect on *E. coli*

All concentrations of gentamicin (6.25, 12.5, 25 μg/ml) inhibited the growth of *E. coli* (G25) considerably in liquid (Figure 3.8 Ci). There was a significant effect of antibiotic treatment on *E. coli* growth (H_4=19.15, P=0.001) with Log_{10} CFU/ml medians of *E. coli* only (8.308), with 6.25 μg/ml gentamicin (7.267), with 12.5 μg/ml gentamicin (7.267), with 25 μg/ml gentamicin (7.268) and for LB only (7.267). There did not appear to be any dose-dependent response to this antibiotic (Figure 3.8 Cii).

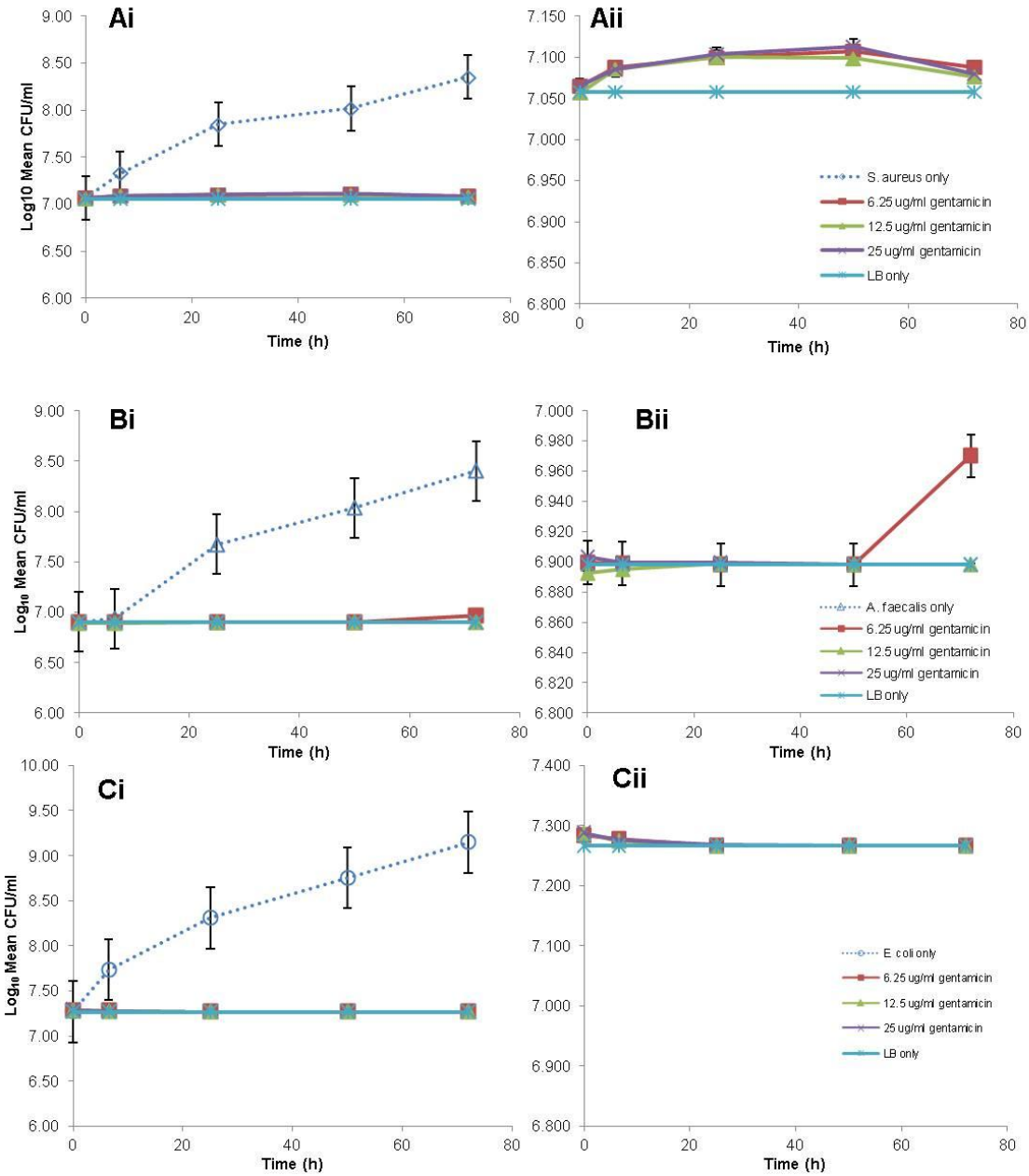

Figure 3.8 Effect of gentamicin (6.25, 12.5 and 25 µg/ml) on MFB in liquid culture. *S. aureus* (G16; **A**), *A. faecalis* (G23; **B**) and *E coli* (G25; **C**), with (**i**) and without (**ii**) bacteria controls. Error bars are ±1 standard error (s.e). N=3 for each treatment and time point.

3.3.4 Effect of Antibiotics on Survival of *P. ovis* mites

3.3.4.1 Microinjection of Antibiotics into *P. ovis* Mites

The delivery of antibiotics directly to the mite gut was investigated by microinjection. The total volume of an average adult *P. ovis* mite was 6.5 μm^3, therefore 65 nl was injected into mites. A number of injection times were tested, however, it was not possible to inject the mite without damaging the cuticle and causing death. This method was therefore not suitable to investigate delivery of antibiotics to the gut of mites and therefore antibiotics were administered in food treatments in *in vitro* chambers.

3.3.4.2 *In vitro* Feeding Experiments Assessing the Effect of Antibiotics on *P. ovis* Survival

Following experiments to investigate the effect of different antibiotics on MFB in both solid and liquid states (Section 3.3.3), antibiotics were then administered to *P. ovis* mites as food to assess the subsequent effect, if any, of disrupting internal bacterial communities on mite survival. Antibiotic concentrations were chosen based on the work of others (Wilkinson, 1998; Douglas *et al.*, 2006; Morimoto *et al.*, 2006; Hardie & Leckstein, 2007; Ben-Yosef *et al.*, 2008) and previous ZOI/liquid assays results.

Pilot Antibiotic *In Vitro* Feeding Assay using Ampicillin and Gentamicin

When mites were setup in chambers (5-10 mites per chamber) and fed water or lamb serum the maximum survival observed was 12 days with both the lamb serum and water diets (Figure 3.9). All mites fed antibiotics were dead by day ten and similar survival curves were seen for the different treatments (Figure 3.9). For all treatments, deaths started to occur after 24 h. All mites had been off-host for the same length of time so it is assumed they had similar levels of food reserves at the start.

Only lamb serum had a significantly better LT_{50} value from the other treatments (Table 3.6). The equality of the curves, however, was not significantly different ($LR_3 = 3.836$, $P = 0.280$) (Crowley & Breslow, 1984), indicating that there was no effect of either gentamicin or ampicillin on mite survival at this concentration (50 μg/ml).

Table 3.6 LT_{50} values for mites fed with gentamicin or ampicillin at a concentration of 50 µg/ml. Values followed by the same letters are not significantly different.

Treatment	Mites alive at start (0d)	Mites alive at end (12 d)	LT_{50} (d)	Standard Error	95% Fiducial CI	
					Lower	Upper
Water	38	0	4.42b	0.25	3.92	4.90
Lamb serum	38	0	6.23a	0.22	5.80	6.68
Ampicillin 50 µg/ml	34	0	5.04b	0.18	4.68	5.40
Gentamicin 50 µg/ml	35	0	4.72b	0.20	4.31	5.13

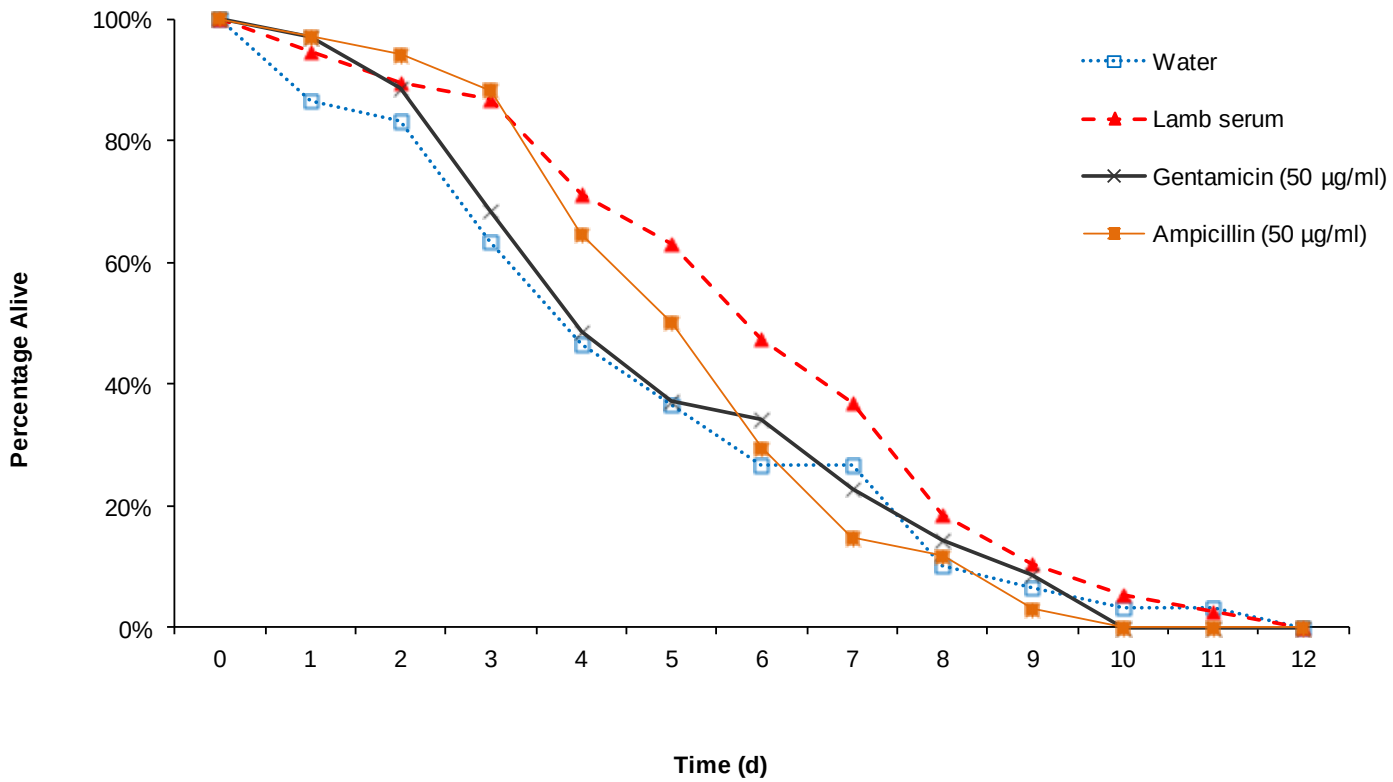

Figure 3.9 Survival curves for *P. ovis* pilot antibiotic assay using ampicillin and gentamicin at 50 µg/ml. Treatments administered (with resulting LT_{50} values) lamb serum (6.32 days), water control (4.42 days), gentamicin (5.04 days) and ampicillin (4.72 days).There were no significant differences in the curves (LR_3 = 3.836, *P* =0.280). Five chambers per treatment, with total mites at start of experiment: Lamb serum (38), water (38), gentamicin (35) and ampicillin (34).

In Vitro Feeding Antibiotic Dose Response Assay

The concentration of antibiotic treatments fed to mites was increased in response to the pilot experiment. In addition, tetracycline replaced ampicillin due to its effects on MFB (Section 3.3.3).

In this experiment all mites were dead by day six, which was half that observed in the first experiment (Section 3.3.4.1), indicating an unusual reponse of all mites to the experiment. Therefore these results were concluded cautiously. As seen in the pilot experiment, however, deaths started to occur after 24 h. The longest survival time was observed in mites fed lamb serum (6 d) (Figure 3.10). A difference in survival curves was seen (LR_4 = 23.12, P <0.001). All mites in the antibiotic treatments were dead by day three in this experiment, half the time observed in the pilot experiment and due to the increased rate of death and therefore lack of later time data points, probit analysis was not suitable as fiducial confidence intervals (CI) for interval estimation could not be formulated (Table 3.7). This indicated that mortality measurements may be more powerful if recorded at more frequent intervals than 24 h.

Table 3.7 LT_{50} values for antibiotic dose response assay. *P. ovis* mites were fed water, lamb serum, gentamicin (100 µg/ml) or tetracycline (100 µg/ml). Missing CI values are due to all mites in antibiotic treatments being dead by day three. Values followed by the same letters are not significantly different.

Treatment	Mites alive at start (0d)	Mites alive at end (6d)	LT_{50} (d)	Standard Error	95% Fiducial CI	
					Lower	Upper
Water	25	0	1.14a	0.13	0.86	1.4
Lamb serum	25	0	2.43b	0.15	2.12	2.72
Gentamicin (100 µg/ml)	30	0	1.87a	7.19	-	-
Tetracycline (100 µg/ml)	30	0	1.89a	16.97	-	-

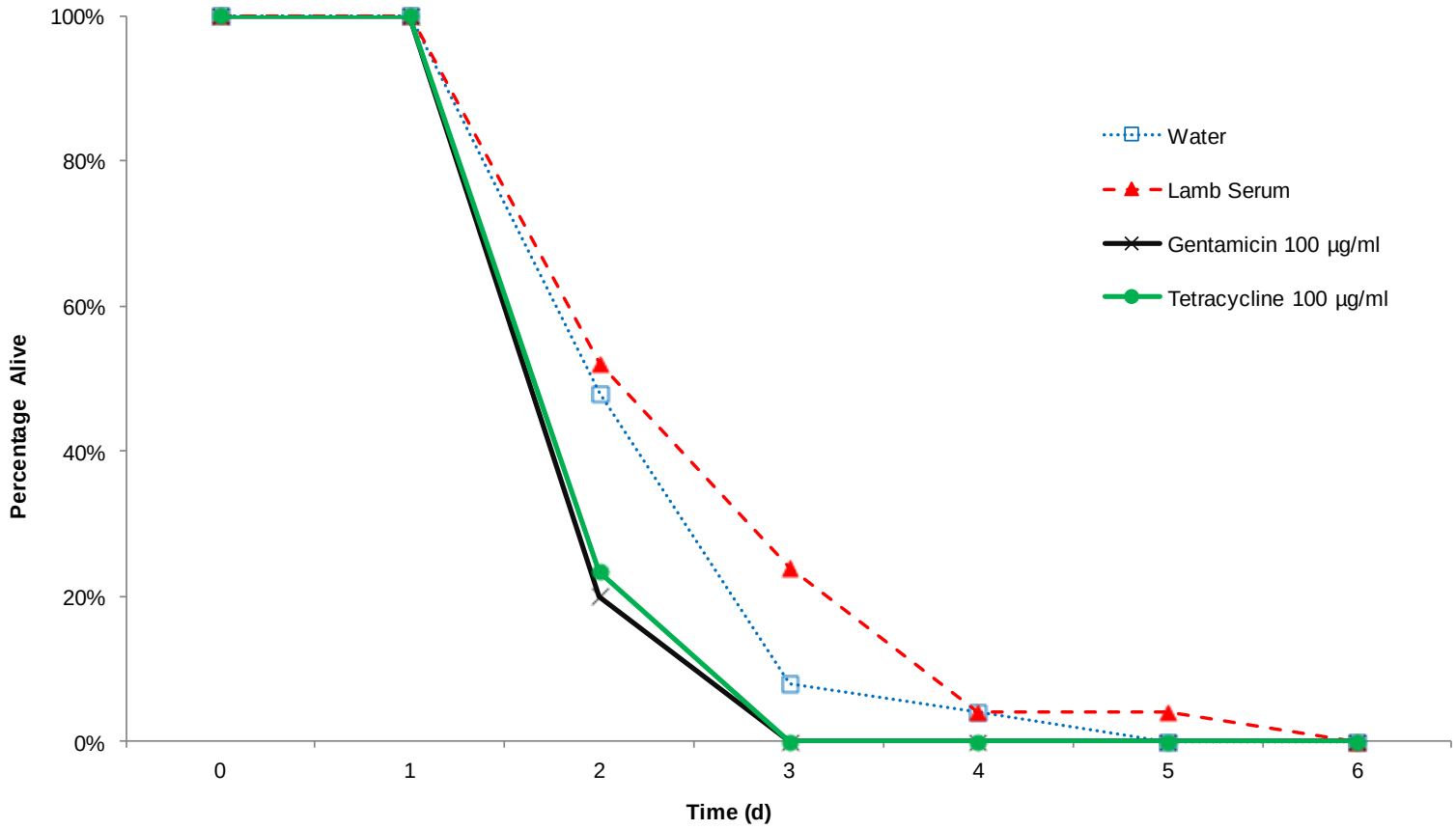

Figure 3.10 Survival curves for *P. ovis* mites fed gentamicin (100 µg/ml) and tetracycline (100 µg/ml) in a dose response assay. Treatments (with resulting LT_{50} values): water (1.14 days), lamb serum (2.43 days), gentamicin (1.87 days) and tetracycline (1.89 days). Significant difference of curves (LR_4 = 23.12, *P* <0.001). Five chambers per treatment with total number of mites at start of experiment: Lamb serum (25), water (25), gentamicin (30), tetracycline (30).

In Vitro Feeding Assay with Diet Enhanced with Antibiotics

In this experiment the antibiotics were dissolved in serum compared to water as used for the previous survival experiments and 10 mites were setup per chamber. The longest survival observed in this experiment was 12 days, by mites fed lamb serum. Mortalities started to occur within the first 24 h except in the water treatment, as seen in all previous survival experiments. The water and lamb serum controls had very similar survival curves until day four when water appeared to have a higher mortality than the lamb serum treatment (Figure 3.11). Although gentamicin-fed mites had a similar curve to water, tetracycline fed mites, however, had a much faster rate of mortality as indicated by the LT_{50} values (Table 3.8) where all mites in this treatment were dead by day nine. The survival curves were significantly different when analysed by log rank (LR_3= 20.251, P <0.001).

Table 3.8 Comparison of *P. ovis* survival when antibiotics were added to lamb serum by LT_{50} values. Gentamicin and tetracycline (100 µg/ml in lamb serum). Values followed by the same letters are not significantly different.

Treatment	Mites alive at start (0d)	Mites alive at end (12d)	LT_{50} (d)	Standard Error	95% Fiducial CI	
					Lower	Upper
Water	57	0	3.80a	0.13	3.55	4.06
Lamb Serum	69	0	5.43b	0.14	5.15	5.71
Gentamicin (100 µg/ml)	57	0	4.94b	0.13	4.68	5.20
Tetracycline (100 µg/ml)	86	0	4.05a	0.12	3.81	4.29

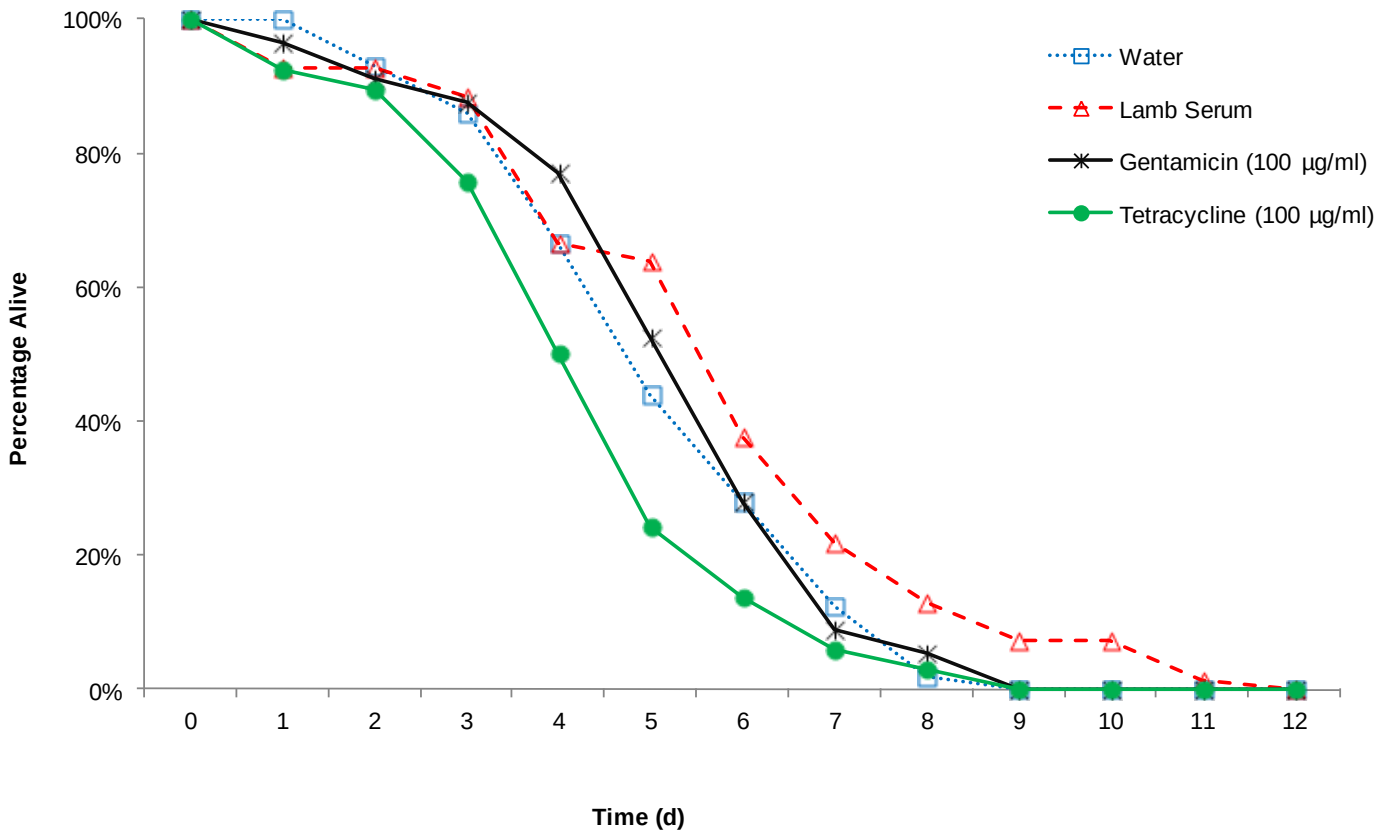

Figure 3.11 Survival curve for *P. ovis* fed lamb serum with added gentamicin (100 µg/ml) or tetracycline (100 µg/ml). Treatments (with resulting LT_{50} values) water (3.80 days), lamb serum (5.43 days), gentamicin (4.94 days), tetracycline (4.05 days). There was a significant difference in curves (LR_3= 20.251, *P* <0.001). Five replicates per treatment with total number of mites at start: Lamb serum (69), water (57), gentamicin (57), tetracycline (86).

Effect of Antibiotics on Mite Bacterial Density- Pilot Experiment

In this experiment, mites were setup in *in vitro* chambers to investigate the effect of antibiotics on *P. ovis* internal bacterial density in addition to survival. A range of bacterial densities were recorded throughout the observation period (12-86 h) (Figure 3.12). The controls, water and lamb serum peaked in density at approximately 61 h. Gentamicin, however, appeared to reduce the bacterial density per mite over time with significant differences due to antibiotic dose (F_1=4.67, P=0.039) but no significant effect of time (F_4=2.15, P=0.099). Tetracycline, however, did not appear to have a negative effect on bacterial density with a trend of increasing bacterial density per mite observed over time.

In addition to bacterial density, in an attempt to determine the quantity of antibiotic that had reached mite internal cavities, a spot lysis assay was carried out. The aim was to produce a cleared area of bacterial lawn indicating lysis by antibiotics from a surface sterilised crushed mite. This ZOI (mm) could then be used to calculate the approximate antibiotic concentration using standard curves of ZOI (mm) versus antibiotic concentration (Appendix 10). The antibiotics chosen, however, could not have targeted all bacteria potentially present in the mite, especially as gentamicin only targets Gram positive bacteria. Because of this, bacterial growth was observed on the spot lysis plates. The diameter of the growths were measured and used to compare treatments. There was a significant effect of antibiotic dose (F_1=9.05, P=0.003) but no significant effect of time (F_5=0.88, P=0.499) on the size of bacterial growth (Figure 3.13).

Interestingly, all treatments produced the largest bacterial growth at 20 h (Figure 3.13) and they all steadily declined after this point. By 86 h, however, there was a significant difference in bacterial growth between the antibiotic and control treatments (F_1=9.05, P=0.003). Bacterial growth at 86 h was comparable to 0 h for water and lamb serum, yet decreased for both antibiotic treatments. This may indicate a required length of time before negative effect of antibiotics is seen (Foster *et al.*, 1986; Diculencu *et al.*, 1998).

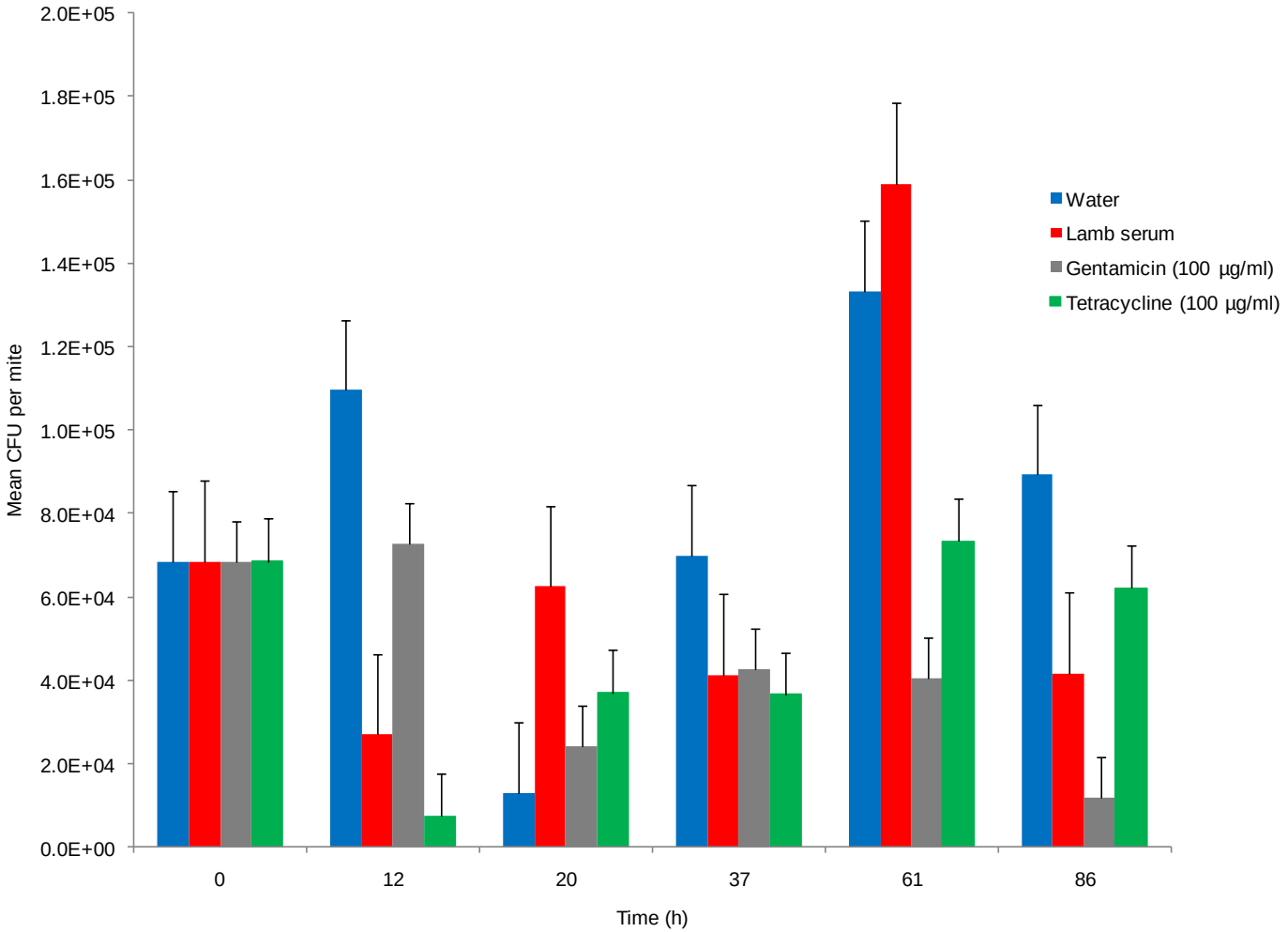

Figure 3.12 Effect of antibiotics on bacterial density in *P. ovis* mites. Significant effect of antibiotic dose (F_1=4.67, *P*=0.039) on mean bacterial density (CFU/mite) but no significant effect of time (F_4=2.15, *P*=0.099). Error bars are sem, N =3 per treatment.

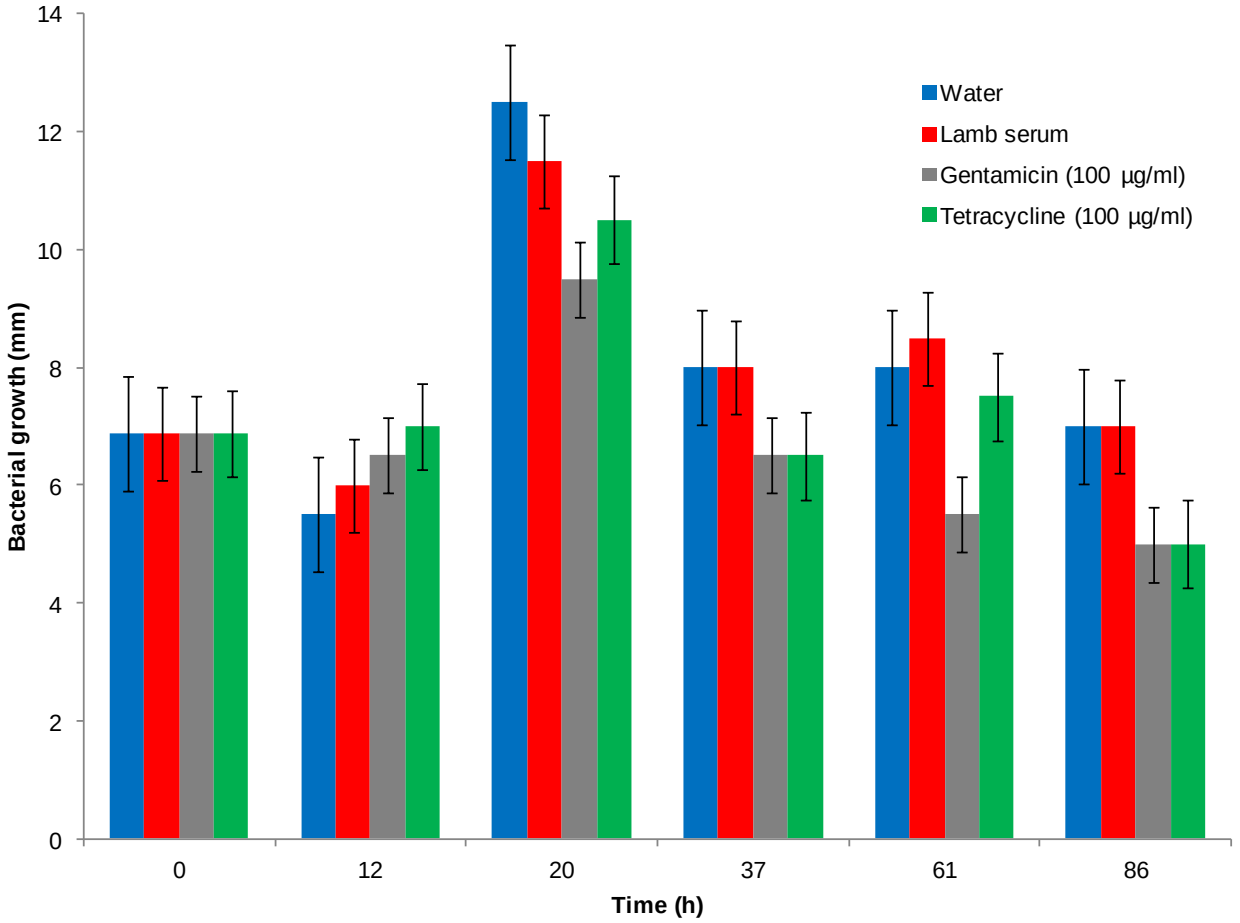

Figure 3.13 Effect of antibiotics on spot lysis assay of bacterial growth from crushed *P. ovis* mites. A significant effect of antibiotic dose (F_1=9.05, *P*=0.003) on bacterial growth was seen but no significant effect of time (F_5=0.88, *P*=0.499). Error bars are sem, N=3 per treatment.

Survival and Bacterial Density Assay

This experiment focused on shorter time intervals between sampling points to try to elucidate effects seen during the first 24 h of the experiment as this was when deaths started to occur in the previous survival experiments. In this experiment some mites remained alive at the end (96 h), yet there were significantly more deaths observed in the tetracycline treatment (Effects =0.7038, $P<0.05$) compared to the lamb serum control when analysed using a GLMM. In this experiment, however, there was not a significant difference between gentamicin (100 μg/ml) and lamb serum control, contrary to all previous survival experiments (Figure 3.14).

When analysed using a GLM, there was a significant effect of status (alive/dead) ($F_1=29.25$, $P<0.001$) and time sampled ($F_8=4.12$, $P<0.001$) on bacterial density but no significant effect of treatment ($F_2=0.96$, $P=0.388$) (Figure 3.15). Regression equations for each treatment is given (Table 3.9). This is a measure of total bacteria, it did not take into account the species of bacteria present.

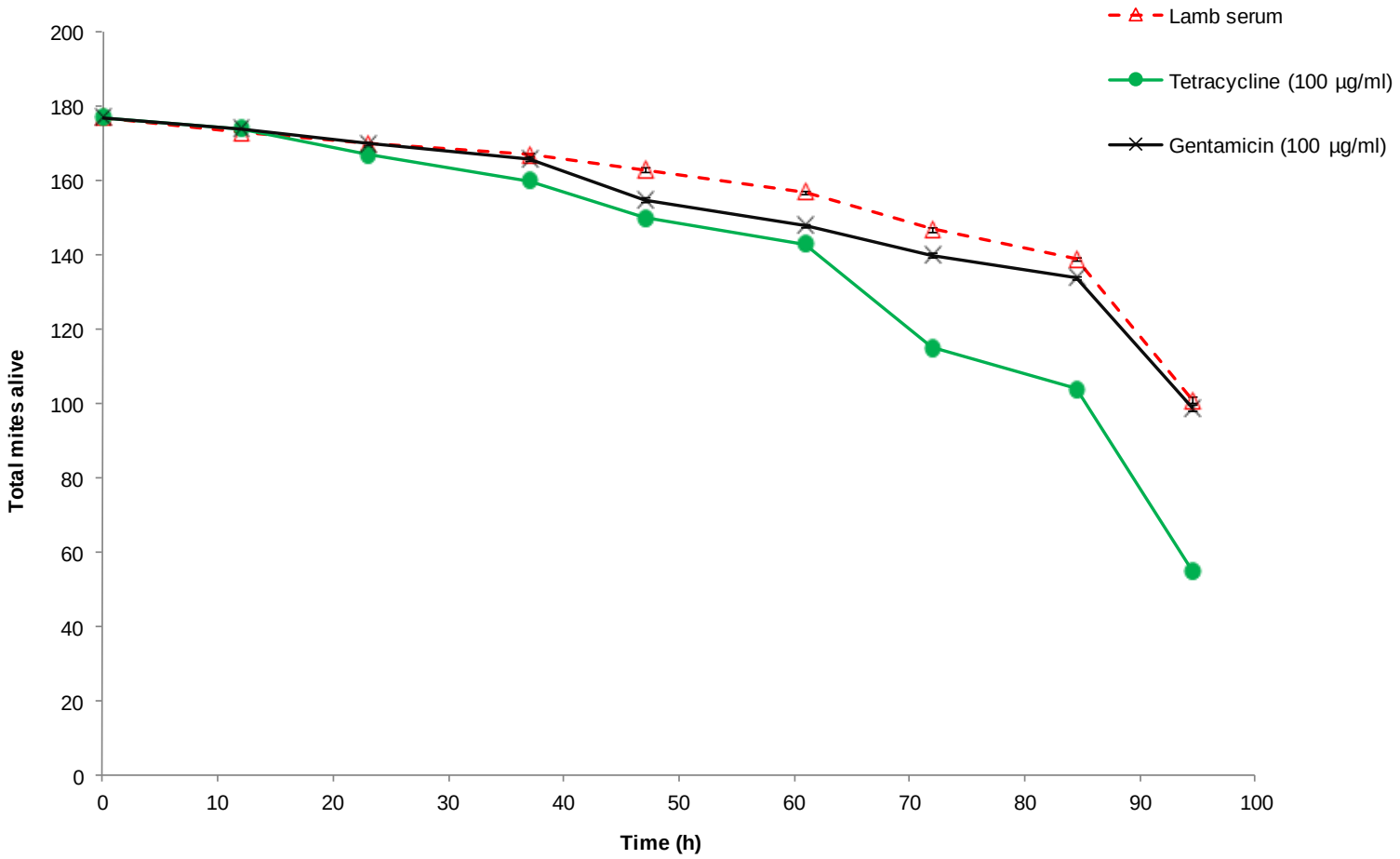

Figure 3.14 Survival curve for *P. ovis* survival and bacterial density assay. Error bars are sem. Food treatments for *P. ovis* mites were lamb serum, gentamicin (100 µg/ml) and tetracycline (100 µg/ml). Both antibiotics were administered in lamb serum. Number of mites at start of experiments = 177 for each treatment.

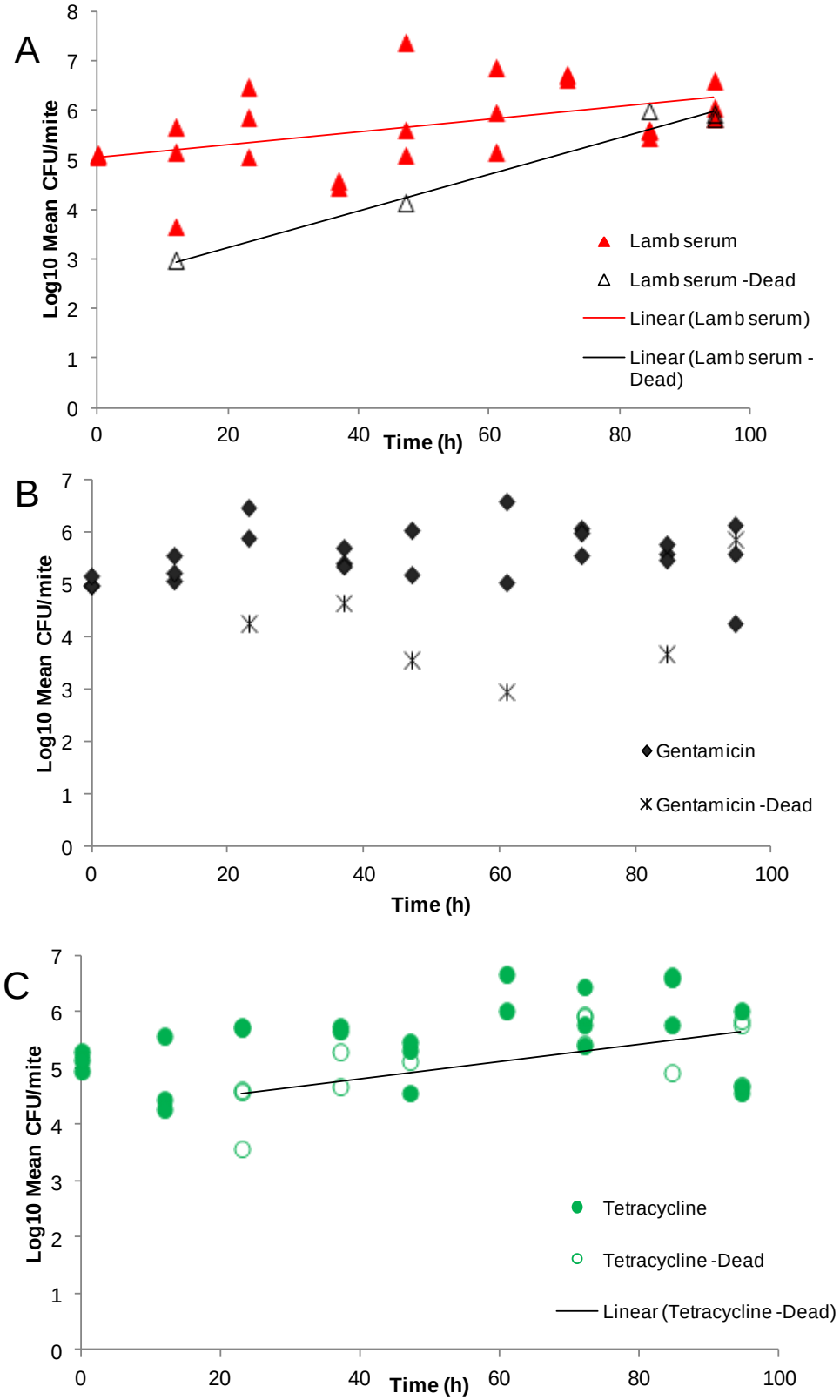

Figure 3.15 Effect on antibiotics on bacterial density of alive/dead *P. ovis* mites. Mites were fed Lamb serum (**A**), Lamb serum with added Gentamicin (100 µg/ml) (**B**) or Tetracycline (100 µg/ml) (**C**). Filled symbols indicate alive mites and open symbols indicate dead mites. Total number of mites, regression equations and *P*-values for trendlines given in Table 3.9.

Table 3.9 Regression equations for bacterial density of *P. ovis* over time fed lamb serum, gentamicin (100 µg/ml) or tetracycline (100 µg/ml). Regression equation: Log_{10} Mean CFU/mite= a(Time(h))+b. R^2 indicates goodness of fit to a linear relationship.

Treatment	Status	N (Total)	a	b	R^2	*P* value
Lamb serum	Alive	24	0.013	5.043	0.231	0.013
	Dead	5	0.037	2.493	0.976	0.002
Gentamicin	Alive	24	0.003	5.381	0.038	0.364
	Dead	6	0.010	3.563	0.076	0.598
Tetracycline	Alive	24	0.008	5.115	0.144	0.068
	Dead	13	0.016	4.176	0.416	0.017

Summary of *P. ovis* Survival Experiments

A range of survival times were seen with the different food treatments with *P. ovis* mites. The minimum, maximum survival and LT_{50} values for each survival experiment has been summarised (Table 3.10). The shortest survival time of 12 h was observed in all treatments in the survival and bacterial density experiment. Other experiments were sampled daily so the shortest survival time was one day. Maximum survival time ranged from three to twelve days. LT_{50} values also ranged from two days (water, dose response assay) to 6.2 days (lamb serum, pilot antibiotic assay).

Table 3.10 Summary of minimum, maximum survival and LT_{50} values for the *in vitro* antibiotic survival assays. Antibiotics were administered in lamb serum (*), all other antibiotics were administered in water.

			Survival		
Experiment	**Treatment**	**N at start**	**Min (days)**	**Max (days)**	**LT_{50}**
Pilot Antibiotic Assay	Water	30	1	12	4.4
	Lamb Serum	38	1	12	6.2
	Gentamicin (50 µg/ml)	35	1	9	4.7
	Ampicillin (50 µg/ml)	34	1	9	5.0
Dose Response Assay	Water	25	2	5	2.0
	Lamb Serum	25	2	6	3.0
	Gentamicin (100 µg/ml)	30	2	3	2.0
	Tetracycline (100 µg/ml)	30	2	3	2.0
Diet enhanced with Antibiotics	Water	57	2	8	5.9
	Lamb Serum	69	1	11	4.8
	Gentamicin (100 µg/ml)*	57	1	8	5.0
	Tetracycline (100 µg/ml)*	86	1	8	4.0
Survival and Bacterial Density	Lamb Serum	177	0.5	3.9	-
	Gentamicin (100 µg/ml)*	177	0.5	3.9	-
	Tetracycline (100 µg/ml)*	177	0.5	3.9	-

3.4 Discussion

3.4.1 Maintenance of *P. ovis* Mites in the Laboratory

Mite chambers constructed for the purpose of maintaining mites in the laboratory were investigated in this chapter. The chambers were designed and constructed identically to enable experimental comparison of treatments applied to the mites. Owing to their small size, the chambers were very practical as many replicates could be kept in similar conditions within incubators. They were also suitable for observations of the mites through the coverslip on the top reducing the disturbance of mites in the internal chamber. One key parameter that was not successfully measured, also owing to chamber size, was the relative humidity inside individual mite chambers. A high relative humidity is important for mite survival by reducing desiccation (Winston & Bates, 1960; Arlian *et al.*, 1981; Smith *et al.*, 1999). This could be overcome in future studies by constructing larger chambers that allowed for internal humidity measurements. Although filter paper provided a reasonable membrane for fluid-food diffusion in this study, it also hardened after successive serum applications. A more porous or mesh-like membrane, may be more suitable for use in the future.

A mean of eight days survival was achieved with the chosen control diet of lamb serum in preliminary experiments. This is comparable to previous efforts of maintaining mites in the laboratory (DeLoach 1984, Rafferty & Gray 1987, O'Brien *et al* 1994, Thind & Ford 2007) (Table 3.1). As this project was concerned with developing a biocontrol method, quick-acting effects on survival were preferential, so this time-frame was thought to be adequate.

Only one feeding experiment was performed with mites received from natural infections of sheep scab disease (maximum survival was 9 days; data not shown) as the time between removal from the host to receipt was unknown for the natural samples which may have affected the condition of individual mites; they were often observed to be desiccated. For this reason they were not included in the experimental analyses. All *in vivo* cultured mites used for the antibiotic feeding experiments had been separated from the sheep host for a maximum 48 h and were never desiccated.

Mites were maintained in chambers at 24°C ±2°C, a much lower temperature than the expected body heat of sheep, which is approximately 39°C (Miller & Monge, 1946).

Higher temperatures were tested for mite survival but were unsuitable as condensation was often produced at the top of the chambers in which mites could get stuck, however, this decrease in temperature may have affected feeding rates.

3.4.2 Determination of *P. ovis* Feeding

3.4.2.1 Microinjection of Antibiotics

To overcome the challenge of determining whether the food and antibiotics were being ingested by the mite, microinjection of the treatments was investigated (Section 3.3.4.1). This technique is often used for transgenic research by injecting DNA into organisms, such as fruit flies (*Drosophila* spp.) (Miller *et al.*, 1987; Jasinskiene *et al.*, 2007). The organisms used are frequently soft-bodied, often in larval stages (Escoubas *et al* 1995). *P. ovis* mites, however, especially during adult stages, have a thickened chitin cuticle, similar to many other mite species (Mehlhorn, 2001). In the present study, penetration of the chitin body wall with the microinjection needle resulted in death, rendering this technique unsuitable to investigate the delivery of antibiotics to the mite gut.

3.4.2.2 ELISA

ELISA was utilised to detect rabbit IgG to determine whether *P. ovis* mites were successfully feeding in the *in vitro* chambers. This was important as it would affect the interpretation of results of the *in vitro* antibiotic feeding experiments.

Rabbit serum was a suitable alternative food to lamb serum as survival times observed for mites were comparable with these two diets. This is not surprising as *P. ovis* mites have been shown to adapt to rabbit hosts after living on sheep hosts (Rafferty & Gray, 1987).

The sandwich ELISA used in this chapter detected rabbit IgG from whole crushed mites, indicating that they fed on this serum. One factor that may have affected the level of sensitivity, which could be improved in future studies, is the small number of mites used for the assay and *P. ovis* may not have ingested large enough quantities of serum for detection. This number was affected by the number kept in the *in vitro* chambers for each treatment, which was very low compared to previous ELISA studies with this organism; Pettit *et al.,* (2000) used 0.5 ml of packed *P. ovis* mites which is a much larger volume than used in this study. Increasing the size of the chambers would allow for a larger number of *P. ovis* mites to be tested.

3.4.3 Antibiotics

3.4.3.1 ZOI Assay

Five different antibiotics (ampicillin, gentamicin, tetracycline, penicillin, chloramphenicol) were tested for their efficacy against mite faecal bacteria (MFB). Of these, most MFB were resistant to ampicillin. Ampicillin is a broad range antibiotic that should inhibit the growth of both Gram positive and negative bacteria, however, it is a member of the penicillin family and resistance has previously been noted to this antibiotic (Cunha, 1992). Gentamicin and tetracycline, however, were much more effective at inhibiting bacterial growth even at low concentrations.

There were two MFB (G17 *Carnobacterium* sp. and G27 Uncultured bacteria) that were resistant to all five antibiotics tested. One hypothesis for this could be the presence of unclassified resistance genes as Riesenfeld *et al.,* (2004) noted that 'soil bacteria are a reservoir of antibiotic resistance genes'. Moreover, *Carnobacterium* are lactic acid bacteria, which have previously been shown to contain resistance genes to antibiotics (Sidhu *et al.*, 2001).

To enable the estimation of antibiotics present within mites after feeding, standard curves of mean ZOI against antibiotic concentration were produced for each antibiotic. When mites fed antibiotics *in vitro* were crushed and plated onto agar, with the aim of measuring ZOI, the presence of other bacteria in *P. ovis* grew on the plates, masking any antibiotic presence. The growth of bacteria was therefore measured and compared between treatments, with mites fed antibiotics having a reduced bacterial growth compared to the lamb serum control. This reduction in bacterial growth also indicates antibiotics were reaching the internal mite cavity to have the effect.

The effect of each antibiotic was compared at 100 μg/ml for each bacterium. This concentration is frequently used to investigate disruption of internal bacteria (Wilkinson, 1998; Douglas *et al.*, 2006; Morimoto *et al.*, 2006; Hardie & Leckstein, 2007; Ben-Yosef *et al.*, 2008), so was appropriate to ascertain the most effective antibiotic against all MFB to use for *in vitro* mite survival experiments. The results also indicated that if 100 μg/ml of antibiotic was administered to mites and as little as 6.25 μg/ml was delivered internally to the mite, this dose could have a significant effect on the microbial community of mites. There were two MFB (G17 *Carnobacterium* sp., G27 uncultured

bacterium 01_44), which were resistant to all five antibiotics tested, but the reason for this is unclear.

3.4.3.2 Antibiotics in Liquid

From the solid agar ZOI assays, tetracycline and gentamicin were consistently within the three most effective antimicrobials. Penicillin, however, was the most effective antibiotic against G16 (*S. aureus*) and G25 (*E. coli*). Penicillin was not used, however, owing to documented cases of resistance of *S. aureus* against this antibiotic (Sabath *et al.*, 1977).

At high concentrations (500 μg/ml) tetracycline did not fully dissolve into the nutrient broth. It was thought that this could affect its efficacy in liquid cultures so as a result this concentration was never used for antibiotic feeding experiments. This may explain why a higher OD was observed with 500 μg/ml tetracycline compared to the lower concentrations.

Gentamicin and tetracycline are both classed as aminoglycosides, yet tetracycline is bacteriostatic whereas gentamicin is bactericidal. In addition gentamicin acts on Gram negative bacteria whereas tetracycline has negative effects on both Gram positive and negative bacteria. The action of gentamicin involves binding to the 30S ribosomal subunit to inhibit protein synthesis and ultimately damages the cell membrane. Tetracycline, however, after binding to the 30S subunit, blocks the attachment of aminoacyl tRNA to prevent new amino acids from attaching to forming peptide (Hahn & Sarre, 1969). The efficacy of the antibiotics may therefore be affected by these mechanisms.

The mite gut is a complex habitat with many bacterial species present, as identified in Chapter 2. The abundance and proportion of these bacterial species within *P. ovis* is however not known. To investigate whether bacterial density affects the efficacy of antibiotics, tetracycline was added to different densities of MFB in liquid (data not shown). The initial bacterial density had a significant affect on the efficacy of antibiotics. This indicates that further knowledge of the composition and dynamics of bacterial communities within *P. ovis* is required to formulate effective microbial control of this disease.

Gentamicin dramatically reduced the growth of all ten MFB tested. Compared to tetracycline, gentamicin was much slower acting, however, the bactericidal effect of this

antibiotic was observed as all growth of G23 and G25 was halted within 24 h. The aim of these experiments was to determine antibiotic effects on MFB separate from *P. ovis* to assess their potential for disrupting bacteria *in vivo*. As quick acting effects of the antibiotics on *P. ovis* bacteria were desired, the liquid experiments were measured over 72 h. No bacterial resistance to antibiotics was observed during this time. It must be noted that antibiotics are not suitable for long term exposure to bacteria due to the risk of resistance to the antibiotics (Bonhoeffer *et al.*, 1997).

These experiments in liquid culture indicated that if at least 6.25 μg/ml of gentamicin was ingested by mites originally administered 100 μg/ml in food, this dose could have a significant effect on the internal microbial community of mites.

3.4.4 *In vitro* Feeding Experiments

3.4.4.1 Pilot Antibiotic *In Vitro* Feeding Assay using Ampicillin and Gentamicin

No significant effect of either antibiotic; ampicillin or gentamicin was observed in this pilot experiment. Firstly the antibiotics used may not have been effective at disrupting the bacterial communities as separate liquid culture experiments with MFB indicated ampicillin was inefficient at killing these MFB. Gentamicin, however, was very efficient under these conditions. Secondly, the antibiotic concentration used (50 μg/ml) may not have been high enough to ensure antibiotics reached the internal cavities of the mite gut. Thirdly, it is possible that the mites did not feed on the water with added antibiotics. Because of these observations, antibiotic concentration in the subsequent experiments was increased and administered in lamb serum.

3.4.4.2 *In Vitro* Feeding of Antibiotics- Dose Response Assay

In this experiment all mites from all treatments (including untreated control) were dead by day six, with all mites administered the 100 μg/ml of antibiotics dead by day three. This reduction in survival compared to the pilot experiment (Section 3.4.4.1) indicates that an increase in antibiotic concentration was required to observe an effect on survival. It is unclear at this stage whether this is due to direct toxic effects of the antibiotic or *via* another mode of action.

3.4.4.3 *In Vitro* Feeding Assay with Antibiotics added to Lamb Serum

Antibiotics were administered in lamb serum in this experiment, to encourage feeding and ingestion of antibiotics. Mites given antibiotics and water were dead by day nine

whereas lamb serum-fed mites survived until day 12. This reduction in survival could have been due to a reduction in feeding in response to the antibiotic or as a result of disruption of the internal bacteria. This second hypothesis was investigated in the subsequent experiment (Section 3.4.4.3).

3.4.4.4 Effect of Antibiotics on Mite Bacterial Density- Pilot Experiment

This experiment investigated the effect of antibiotics on the internal bacterial density of mites. Before plating, mites were surface sterilised to ensure bacteria were of internal origin, not external contamination. After 20 h there was a significant reduction in bacteria in all treatments except the lamb serum control. There was a peak in bacterial density at 61 h with both water and lamb serum treatment. By 86 h, gentamicin-fed mites had a significantly reduced bacterial density compared to lamb serum control. Although tetracycline appeared to significantly reduce bacterial density within the first 20 h, this 'recovered' over the experiment. This experiment did not discriminate the bacterial species present, only total abundance, which would be necessary to estimate effects on targeted species and communities.

Surface sterilised whole crushed mites were also plated onto pre-inoculated agar plates to measure the amount of antibiotic ingested by the mite, although this objective was not possible due to the growth of other bacteria present within the mite. This bacterial growth was measured over time as an alternative comparison of the effects of each antibiotic. This presence of bacterial growth indicated that there were bacteria present within the mites that could grow in addition to, or even outcompete, the bacteria on the pre-inoculated NA plate. There was great variation between treatments over time with a minimum log 4 CFU per mite and a maximum of log 5.2 CFU per mite measured, with lamb serum and water-fed mites exhibiting the largest bacterial growth except at 20 h. By 86 h there was a significant difference in bacterial growth observed with antibiotic-fed mites, which exhibited much lower bacterial growth compared to lamb serum and water fed controls.

3.4.4.5 Survival and Bacterial Density Assay

Gentamicin-fed mites in this experiment did not have a reduced survival time compared to controls, which highlights the sensitivity of mites living *in vitro*. Mites fed tetracycline did show a reduced survival as observed in previous experiments.

Over 96 h the bacterial density increased for both live and dead mites, yet live mites were higher overall. This could be due to effects on bacterial communities post-death before analysis, although sampling was every 12 h to overcome this. The two antibiotics used (gentamicin and tetracycline) are not effective against all bacteria present within *P. ovis*, as observed from MFB experiments (Section 3.3.3, Table 3.5). These results demonstrate the complex bacterial interactions within *P. ovis*. The destruction of bacteria susceptible to the antibiotics may have resulted in otherwise suppressed bacteria to proliferate (Torsvik *et al.*, 1990).

These experiments did not analyse how the specific composition of bacteria within *P. ovis* mites were affected by administration of antibiotics. Techniques such as real-time PCR (Bustin *et al.*, 2009) could be employed to investigate the abundance of specific bacterial species in response to antibiotic treatments for both internally and excreted faecal bacteria.

3.4.4.6 General Conclusions from *in vitro* Antibiotic Experiments

- These *in vitro* feeding experiments could not determine whether the antibiotics used were directly toxic to *P. ovis*. Previous studies observed that tetracycline inhibited egg hatching through the suppression of *Wolbachia*, but there were no direct toxic effects on vine weevils, *Otiorhynchus sulcatus* (Son *et al.*, 2008) or on filarial worms, *Brugia pahangi* and *Dirofilaria immitis* (Bandi *et al.*, 1999). To test toxicity of antibiotics, Dedeine *et al.,* (2001) assessed a closely related species that did not harbour the symbiont studied. The presence of symbionts within *P. ovis* or *P. cuniculi* is still unclear but this method could be utilised in the future.

- The feeding experiments were not able to observe or measure the effect of antibiotics on growth, development or reproduction of *P. ovis*. Previous studies investigating commensal/symbiont removal focused on these life history factors (Wilkinson, 1998; Morimoto *et al.*, 2006; Hardie & Leckstein, 2007) but this was not possible with the current *in vitro* chambers.

- Adult mites were used for the *in vitro* feeding experiments as this stage was investigated for microbial community composition in Chapter 2, however, the timing of antibiotic application may affect the response observed, for example

Nogge & Gerresheim (1982) found that effects of antibiotic on flies were smaller the older the flies were.

- Water was used as a 'no-food' control in the feeding experiments. Water was required to maintain humidity in the chambers but may not strictly be 'no food' treatment as *P. ovis* have been shown to feed on water (Sinclair & Filan, 1989).

- Future experiments could administer a mix of antibiotics to observe the effect of disrupting a greater range of bacterial types or antibiotics that target specific bacteria, such as *Comamona*s sp., identified in Chapter 2

3.4.4.7 Summary

- Mite chambers provide adequate short-term maintenance of *P. ovis* mites but further investigation is required to produce long-term maintenance off-host, especially to produce suitable reproductive conditions for the mites.

- *P. ovis* have a complex community of internal bacteria, with some showing resistance to a number of antibiotics.

- Some antibiotics appeared to reduce the internal bacterial density of *P. ovis* in this study.

4 Bacteriophage Isolation and Characterisation

4.1 Bacteriophage Introduction

4.1.1 History of Bacteriophage Therapy

Since their discovery in 1915, bacteriophage (viruses of bacteria) and their use in therapy have been under debate. They have colonised every conceivable habitat (Wommack *et al.*, 2009) and their specific bacteriocidal activity makes them a valuable resource for the treatment of bacterial infections (Hanlon, 2007). Research on bacteriophage began during the 1920s, mainly in Eastern Europe. Currently a number of Western companies are developing, licensing and selling bacteriophage-based products, such as BigDNA (bacteriophage DNA vaccinations), Biophage Pharma Inc. (environmental therapies and diagnostics) and Intralytix (food safety) (Housby & Mann, 2009). The advantages and limitations of bacteriophage therapy are reviewed in detail in (Summers, 2001; Hausler, 2006; Skurnik & Strauch, 2006; Abedon, 2010) and summarised in Chapter 1, Table 1.3.

There were a number of challenges facing early bacteriophage research including poorly recorded experimental trials and anecdotal qualitative evidence as well as language barriers; most bacteriophage research occurred in either Poland or Russia (Hanlon, 2007). The first controlled clinical trials were carried out by Smith and Huggins (1982) with work on *Escherichia coli* infections in mice which although largely successful, identified obstacles with *in vivo* application, such as effect of pH and temperature on bacteriophage preparations.

Bacteriophage have been investigated for use against bacterial foodborne pathogens such as *Salmonella,* in cheese making (Modi *et al.*, 2001) and chicken skins (Goode *et al.*, 2003), *Staphylococcus aureus* in curd making (Garcia *et al.*, 2007) and *Listeria monocytogenes* (Leverentz *et al.*, 2003) for which a commercial product (LISTEX) is now available (EBI Food Safety, 2011) and has also received the GRAS (generally regarded as safe) status for use in all food products (Hagens & Loessner, 2010).

Agricultural uses have been targeted including *E. coli* infections in calves (Smith *et al.*, 1987) and chickens (Barrow *et al.*, 1998), *coliobacillosis* in broiler chickens (Huff *et al.*, 2004) and *S. aureus* infections causing mastitis in lactating dairy cattle (Gill *et al.*, 2006).

Bacteriophage have been assessed for use against antibiotic and drug resistant bacteria (Barrow *et al.*, 1998; Biswas *et al.*, 2002) as well as a host of others including respiratory infections in mice (Nelson *et al.*, 2001), *Pseduomonas* infections in fish (Park *et al.*, 2000),

protection against skin graft destruction by bacteria (Soothill, 1994) and wastewater treatment (Barrow *et al.*, 1998; Withey *et al.*, 2005). Douglas (1989) suggested the potential of pest control through disruption of their symbionts. This idea of using bacteriophage to eliminate arthropod symbionts for removal of the arthropod host is in a current patent (Patent number WO 03/061391 A1) (Blackwell & Wellburn, 2003).

4.1.2 Bacteriophage Properties suitable for use in Bacteriophage Biocontrol

For a specific bacteriophage to be suitable for use in bacteriophage biocontrol or therapy, a number of requirements must be satisfied:

The bacteriophage must be productive (lytic) so that bacterial cells are destroyed in the bacteriophage replication process (Nelson *et al.*, 2001; Abedon, 2008). Furthermore, lysogenic (temperate) bacteriophage which do not kill bacteria by lysis should be avoided for a number of reasons. Some bacteriophage are capable of lysogenic conversions whereby bacteriophage DNA is integrated into the genome of the bacterium they infect. This process can result in increased virulence of the bacterium, especially if the bacteriophage genes encode bacterial virulence factors (Gill & Hyman, 2010). Some lysogens can become immune to infection by the same bacteriophage and some are capable of generalised transduction; a process involving the mass movement of DNA between hosts (Gill & Hyman, 2010).

Chosen bacteriophage must be safe towards humans and animals. Although there has been no evidence of negative side affects on mammals thus far (Weber-Dabrowska *et al.*, 1987; Bruttin & Brussow, 2005; Brussow, 2005; Dabrowska *et al.*, 2005; Mann, 2008; Wright *et al.*, 2009), regulatory tests are required before use commercially (Gorski & Weber-Dabrowska, 2005). For use against sheep scab mites, regulatory approval would be sought through the Health and Safety Executive, which would include mammalian toxicity tests as well as efficacy tests against mites *in vivo*. Importantly the biology of the specific bacteriophage needs to be understood and the bacteriophage receptor on the bacterial cell surface should be characterised (Skurnik & Strauch, 2006).

The specificity of the bacteriophage is important. For example, a poor candidate for bacteriophage therapy is T4 bacteriophage, as it has a very narrow infective range. This is unfortunate because T4 has been sequenced and widely characterised; two essential requirements for regulatory tests (Bruttin & Brussow, 2005). Alternatively, a polyvalent

bacteriophage, which is able to infect a number of strains within a bacterial species may be more appropriate (Brussow, 2005). Moreover, mixtures or 'cocktails' of bacteriophage are formulated with the aim of improving efficacy through synergistic action of bacteriophage with differing specificities or to overcome bacterial resistance (Goodridge & Abedon, 2003; Garcia *et al.*, 2009).

For industrial and commercial purposes, the bacteriophage should be able to be produced cost-effectively on a large scale (often in fermenters) and retain infectivity after storage. A number of methods for stabilisation are possible including lyophilisation (freeze-drying), which can produce bacteriophage that are more stable, smaller and easier to apply, without any loss in activity (Brussow, 2005).

A number of application methods in mammals have been investigated depending on the target bacterium including intra-nasal (Barrow *et al.*, 1998), intra-muscular (Huff *et al.*, 2004), impregnated patches (Stone, 2002) and aerosols (Leverentz *et al.*, 2003; Garcia *et al.*, 2009).

4.1.3 Methods for Bacteriophage Identification

Although 'no universal classification' exists (Clokie & Kropinski, 2009), there are three main methods for identifying bacteriophage:

1. Morphology, as visualised by transmission electron microscopy (TEM) including virion shape and dimensions (Barrangou *et al.*, 2002; Gill *et al.*, 2003; Ackermann, 2006).
2. Infection phenotype, including the host range and growth properties (Abedon, 2008).
3. Genome sequence-based analyses (Rohwer & Edwards, 2002) such as restriction fragment length polymorphisms (RFLP) (Gill *et al.*, 2003; Abedon, 2008), pulsed field gel electrophoresis (PFGE) (Jiang *et al.*, 2003) and PFGE with pyrosequencing (Yasmin *et al.*, 2010). Restriction enzyme (RE) digests are especially useful as they can be used to compare bacteriophage genomes by producing physical cleavage maps (Hamlett *et al.*, 1977) as well as determination of genome type, as only dsDNA is digested by REs (Peters, 2009).

4.1.4 Difficulties of Bacteriophage Isolation

Endosymbionts are generally unculturable outside of their host owing to the problems of recreating the host environment *in vitro*. This presents a significant challenge if bacteriophage are to be isolated against unculturable bacteria, such as obligate endosymbionts, as the double agar method of bacteriophage isolation requires co-culturing of bacteriophage and bacteria *in vitro* (Adams, 1959). There are limitations to isolating bacteriophage from the environment directly, so enrichment techniques, addition of the bacterial host required for bacteriophage replication, are often employed. Although successful, this method can be selective towards bacteriophage that propagate under particular experimental conditions (Van Twest & Kropinski, 2009). Although bacteriophage are most commonly found in the same environmental area as their host bacteria, they may be specific to a particular strain of bacteria. Additionally the natural bacteriophage environment may protect and stabilise the bacteriophage which may become inactivated once removed from it. For example, *Erwinia amylova* bacteriophage can be affected by UV light and desiccation but are protected from this damage when residing in the soil (Gill *et al.*, 2003; Abedon, 2008).

4.1.5 Bacteriophage Growth Parameters

A wealth of research has been carried out into the ecology of bacteriophage (Stephenson, 2003; Abedon, 2008; Clokie & Kropinski, 2009) and their growth and replication dynamics have been documented (Ellis & Delbruck, 1939; You *et al.*, 2002). Bacteriophage growth can be summarised into four stages: Initiation, injection, multiplication and release (Chapter 1, Section 1.4.2). It is important to determine these basic growth parameters of experimental bacteriophage. As with all organisms, bacteriophage growth is affected by resource availability, in this case actively growing bacteria (Abedon, 2008; Garcia *et al.*, 2009; Poisot *et al.*, 2011) and the lysis of bacterial cells can be affected by temperature, pH and culture conditions (Krueger, 1930).

The spatial structure in which a bacteriophage exists will affect its potential interactions with bacteria. In this chapter, infectivity of bacteriophage was investigated in a spatially structured environment of semi-solid agar, in which diffusion and progeny release occurs on a local level (Abedon, 2008). In addition, liquid media which provides a more

homogenous environment, was used in an attempt to mimic the potential environment within *P. ovis* internal cavities.

4.1.6 Summary and Aims

The work described in this chapter aims to investigate the potential of using bacteriophage infective for *P. ovis*-associated bacteria. To do this, experiments were undertaken to:

- isolate bacteriophage infective against *P. ovis*-derived bacteria from environmental samples;
- characterise isolated bacteriophage through growth dynamics, plaque morphology, TEM observations and responses to chemical, temperature and enzymes;
- determine the effect of isolated bacteriophage on the survival and bacterial density of *P. ovis* mites.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Chemicals and Microbiological Media

All chemicals used were of molecular grade and sourced from Sigma (Sigma Chemical Co., UK), unless stated otherwise. Microbiological media; nutrient agar (NA) (CM0003), plate count agar (PCA) (CM0325), nutrient broth (NB) (B00210) and maximum recovery diluent (MRD) (CM0733) was supplied from Oxoid (Oxoid Ltd., UK) and made up according to manufacturer's instructions.

4.2.1.2 Environmental Samples for Bacteriophage Isolation

Environmental samples were collected from a variety of sites (Table 4.1) throughout Scotland and kept at 4°C until used for bacteriophage isolation.

4.2.1.3 Mite Faecal Bacteria

Ten bacteria were used for bacteriophage isolation which were originally isolated from *P. ovis* faecal trails (Section 2.3.2) and identified by DNA sequencing (Table 2.7) and were maintained as previously described (Section 2.2.2.5).

Table 4.1 Location and type of environmental sample collected for bacteriophage isolation. Scab-infected sheep fleece was received from natural sheep scab infections (S) or *in vivo* culture (M) (see Section 2.2.1.2).

Environmental sample	Brief description of origin	Location
W1	Mud (water hole) Woodhouselee	Midlothian
W2	Water (water hole) Woodhouselee	Midlothian
W3	Water from trough Woodhouselee	Midlothian
W4	Sheep faeces Woodhouselee	Midlothian
W5	Scab-infected sheep fleece (S38)	St Boswells
S39	Scab-infected sheep fleece (S39)	St Boswells
W7	Mud from Easter Bush	Midlothian
W8	Scab-infected sheep fleece (S8)	Dumfries
W9	Scab-infected sheep fleece (S27)	Dumfries
W10	Mud from Woodhouselee	Midlothian
W11	Mud from wood at Easter Bush-(wet)	Midlothian
S33	Scab-infected sheep fleece (S33)	Dumfries
S34	Scab-infected sheep fleece (S34)	Dumfries
M6	*P. ovis* mites (M6)	*In vivo* culture
S36	Scab-infected sheep fleece (S36)	Perth
S40	Scab-infected sheep fleece (S40)	Thurso
S44	Scab-infected sheep fleece (S44)	Inverness
M7F	Scab-infected sheep fleece (M7)	*In vivo* culture
D1	Sheep pen soil	Aberdeenshire

4.2.2 Methods

4.2.2.1 Bacteriophage Isolation: Enrichment Culture Technique

A 1 g sub-sample of each environmental sample (Table 4.1) was added to a sterile 50 ml falcon tube and mixed with 1 ml of MFB overnight liquid culture (Section 2.2.2.5) and the volume made up to 30 ml with NB. Samples were then incubated overnight on an orbital shaker at RT. After incubation, the culture was centrifuged at 3000 g for 20 min to sediment excess bacteria and environmental sample. The supernatant was removed and filtered through a 0.45 μm syringe filter (Whatman) into a sterile tube and stored at 4°C until use.

4.2.2.2 Bacteriophage Plating: Double Agar Method

Bacteriophage (either an enrichment culture for bacteriophage isolation (Section 4.2.2.1) or bacteriophage isolate for purification (Section 4.2.2.4) or high-titre lysate (Section 4.2.2.5)) were plated using the double agar method (Adams, 1959). To a sterile tube, 100 μl of enrichment culture (Section 4.2.2.1) or bacteriophage dilution (Section 4.2.2.4 and 4.2.2.5) and 100 μl of specific MFB liquid overnight culture were mixed and incubated for 30 min at RT. To this, 3.5 ml of molten top agar (NB + 0.06% agar) cooled to 47°C was added, mixed gently then poured on top of a hardened NA plate. The top agar was allowed to set then plates inverted and incubated overnight at RT.

4.2.2.3 Bacteriophage Selection: Plaque Picking

Cleared areas of bacterial lawn or lytic plaques indicated the presence of bacteriophage. These plaques were removed using a wide-bore pipette tip and placed in 1 ml of phage storage buffer (PSB; 1 M Tris HCl pH 7.4, 166 M $MgSO_4$, 2% gelatine). The plaque was left to soak out for 2 h at RT. The soak-out was then filter sterilised and stored at 4°C.

4.2.2.4 Bacteriophage Purification

After isolation, picked plaques were purified by a minimum of three rounds of plating on NA (Section 4.2.2.2) and plaque picking (Section 4.2.2.3) to ensure pure bacteriophage isolates. Bacteriophage isolates were named by location of environmental sampling and bacterial host infective against for example. G25W1 is infective against G25 (MFB host) and was the first bacteriophage isolated from W (Woodhouselee Farm).

4.2.2.5 Production of High Titre Bacteriophage Lysates

For experimentation and storage, high-titre bacteriophage lysates were produced. Bacteriophage plaques were purified as described previously (Section 4.2.2.4) and ten-fold serial dilutions plated using the double-agar technique (Section 4.2.2.2) to produce confluent lysis (where plaques are just touching). Five ml PSB was added to the plates and incubated at RT for several hours on an orbital shaker (or at 4°C O/N). The PSB was then removed from the plates and transferred to a sterile tube. A further 1 ml of PSB was added and the plates then tilted at 30° to encourage accumulation of the buffer. The remaining buffer was then removed and added to the same tube. The PSB/high-titre lysate was centrifuged at 1398 x *g*, the supernatant removed and filtered using a 0.45 μm filter (33 mm Millipore). This high-titre lysate was stored at 4°C.

4.2.2.6 Glycerol Stocks of Bacteriophage

An aliquot of bacteriophage high-titre lysate (Section 4.2.2.5) was stored in 25% sterile glycerol at -20°C.

4.2.2.7 Spot-lysis Assay

Spot-lysis assays were used to assess bacteriophage activity against bacterial hosts as an alternative to the double-agar technique. To 3.5 ml of molten NB top agar (cooled to 47°C), 100 μl of appropriate MFB was added, mixed gently and poured onto NA plates to set. Once the top agar was solidified, 5 μl ten-fold serial dilutions of bacteriophage were spotted onto the agar and allowed to dry. Plates were then inverted and incubated at 27°C overnight.

4.2.2.8 Bacteriophage Characterisation

Bacteriophage Cross-reactivity Assay

Each isolated bacteriophage was tested for its infective ability against all other MFB (Poisot *et al.*, 2011). This was achieved using the spot-lysis assay (Section 4.2.2.7). Each bacteriophage lysate was tested in duplicate against each MFB. After incubation, the type of bacteriophage activity was recorded as lytic, lysogenic or no infection.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was utilised to observe the ultrastructure of bacteriophage for identification. Bacteriophage was plated using the double layer technique (Section 4.2.2.2) at a dilution that produced separate and distinct plaques. A

single plaque was picked and stored in 1 ml of sterile distilled water. The bacteriophage samples were prepared for TEM at The Electron Microscope Laboratory, Edinburgh University. TEM images were collected using a Philips (FEI) CM120 Biotwin transmission electron microscope.

DNA Extraction from Bacteriophage

DNA extraction method was based on Rohwer *et al.,* (2002). Briefly, 10 ml of high-titre bacteriophage lysate (Section 4.2.2.5) was incubated with 10 μg/ml ribonuclease A and 0.25 SU/ml deoxyribonuclease 1 (DNase) for 1 h at RT. The sample was centrifuged for 30 min at 5590 x *g*, the supernatant removed and added to 10 ml polyethylene glycol (PEG 8000; 10% solution) and incubated overnight at 4°C. The PEG-bacteriophage precipitate was collected by centrifugation at 4528 x *g* for 30 min using a Sorvall GSA rotor, the supernatant discarded and the resultant pellet re-suspended into 1 ml PSB. To this, 5 μl of Proteinase K (20 mg/ml) and 500 μl of SDS (2%) were added and the mixture incubated at 58°C in a water bath for 1 h. The mixture was then allowed to cool to RT. An equal volume of phenol was added and the tube inverted several times, then spun at 503 x *g* for 5 min. The aqueous phase was transferred to a fresh 2 ml tube using a wide-bore pipette. An equal volume of phenol:chloroform (1:1) was added and inverted several times to mix. The sample was then spun at 503 x *g* for 5 min and the aqueous phase transferred to a fresh 1.5 ml tube. This process was then repeated, once using phenol:chloroform (1:1) and once with chloroform only. The aqueous phase was transferred to a fresh 1.5 ml tube after every spin. Two volumes of ice-cold isopropanol were then added, mixed by inversion and incubated overnight at -20°C. The mixture was spun at 10,956 x *g* for 20 min and the isopropanol removed. To the tube, 1 ml of 70% ethanol was added and spun at 10,956 x *g* for 2 min. This ethanol wash step was repeated and as much ethanol as possible was removed using a pipette without disturbing the DNA pellet. The tube was then left open in a laminar flow hood for approximately 30 min for any remaining ethanol to evaporate. The DNA pellet was finally resuspended in 50 μl sterile distilled water and the amount of DNA measured using a ND-1000 spectrophotometer (Nanodrop). The bacteriophage DNA was checked by running on a 0.7% agarose gel containing GelRed (Biotium) in TBE buffer with a 1 Kb^+ ladder (Invitrogen) for size calibration. Gels were run at 100 V and visualised with a ChemiImager (Alpha Innotech Corp).

Restriction Enzyme Digests

Extracted bacteriophage DNA (Section 4.2.2.8) was purified using High Pure PCR product purification kit (Roche) following manufacturer's instructions. DNA (15 µl bacteriophage or 2 µl lambda) was then digested with a number of restriction enzymes (RE) (Table 4.2). All RE mixtures were set up in duplicate, following manufacturer's recommended conditions (Promega) and a lambda (λ) DNA control was included. For *AluI* and *HinfI* the mixtures were incubated at 37°C for 4 h and for *Taq1* incubation was for 2.5 h at 65°C. RE digest or uncut bacteriophage DNA (8 µl) was run on a 2% agarose gel containing GelRed (Biotium) in TBE buffer (Eurogentec) with 100 bp and 1 Kb^+ ladder for size calibration (Invitrogen). Gels were run at 125 V and visualised using a ChemiImager (Alpha Innotech Corp).

Table 4.2 Restriction enzymes digest of bacteriophage DNA. Restriction enzyme and specific cleavage sites.

Restriction Enzyme	DNA sequence with enzyme cleavage site (^)
HinfI	G^ANTC
	CTNA^G
AluI	AG^CT
	TC^GA
TaqI	T^CGA
	AGC^T

Chemical and Enzymatic Treatment of Bacteriophage

All chemical and enzyme tests on bacteriophage used 100 µl of high-titre bacteriophage lysate (Section 4.2.2.5; approximately Log10 PFU) mixed with 900 µl of the test chemical and incubated (as detailed in Table 4.3) (Steinberg *et al.*, 1976; Rigby *et al.*, 1989; Capra *et al.*, 2004). Treated bacteriophage were then plated using the spot-lysis assay (Section 4.2.2.7). A non-treated bacteriophage control and treatment only control was always included. The effect of each treatment on bacteriophage plaque formation was compared to the non-treated bacteriophage control and recorded as follows:

Plaques present (+), plaques present- reduced compared to controls (<+), plaques absent (-) or direct effect of treatment on bacteria (L).

Table 4.3 Chemical and enzymatic treatments used to test isolated bacteriophage.

Chemical treatments	Incubation time and temperature
SDS (1%)	37°C for 1 h
Triton-X-100 (1%) Chloroform Ethanol (70%) Isopropanol EDTA (0.01M)	RT for 1 h
Enzymes	
Proteinase K Trypsin Lysozyme DNase	37°C for 1 h

Effect of Temperature on Bacteriophage Plaque Formation: Heat/Cold Treatment

One ml of each bacteriophage lysate was incubated at either -20°C for 1 h and thawed (repeated three times) or heated at 60°C in a water bath for 1 h. Bacteriophage were then plated using the spot-lysis assay (Section 4.2.2.7) to assess the effect of temperature on plaque formation as compared to an untreated control.

Effect of pH on Bacteriophage Plaque Formation

Bacteriophage buffer (PSB) was adjusted to different pHs (2, 3, 4, 5, 6, 7, 8, 9, 10.5 and 12) using either 2 M Hydrogen Chloride or 1 M Sodium Hydroxide. A mixture of 100 µl of bacteriophage lysate and 900 µl of pH-adjusted PSB was incubated at 37°C for 1 h (Capra *et al.*, 2004). Bacteriophage-PSB mixtures were then plated using the spot lysis assay (Section 4.2.2.7) and plaque-formation assessed as compared to an untreated control.

4.2.2.9 *In Vitro* Bacteriophage and Host Assays

Mite Faecal Bacteria Growth Curves

MFB overnight liquid culture (100 µl with CFU of approximately Log8) (Chapter 2, Section 2.2.2.5) was inoculated into 30 ml of NB and incubated with shaking at 27°C. Replicates were setup to allow individual time-point sampling. At 0, 1-12 (hourly), 15, 18, and 24 h, 1 ml of culture was removed and optical density (OD) at 600 nm was measured (Biophotometer, Eppendorf). In addition, at each time point, 1 ml was removed and serially diluted in 9 ml of MRD and 100 µl of three appropriate ten-fold serial dilutions, plated on PCA in duplicate. Plates were incubated at 27°C overnight and viable colonies counted per plate to calculate Colony Forming Units (CFU/ml) to produce standard curves (Appendix 9).

Bacteriophage Activity in Liquid Culture

The effect of isolated bacteriophage against MFB in liquid phase was investigated. MFB overnight liquid culture (100 µl with CFU of approximately Log8) (Section 2.2.2.7) was inoculated into 10 ml NB. To this 100 µl of a single bacteriophage lysate or a mixture of bacteriophage (cocktail) were added at different dilutions to produce multiplicities of infection (MOI) (0.5, 1, 10, 20, 50), in triplicate. Negative controls of LB only and bacteria only were also set up. Tubes were incubated with shaking at 27°C. At time points over 24 h, 1 ml of the mixture was removed, OD (600 nm) measured (Biophotometer, Eppendorf), which was converted to Log_{10} mean CFU/ml using MFB-specific standard curves (Appendix 9).

Effect of Bacteriophage on *In Vitro* Mite Survival

To test the effect of bacteriophage on *P. ovis* survival, an initial experiment of feeding bacteriophage to *P. ovis* in *in vitro* chambers was carried out as previously described (Section 3.2.2.5) to test the toxicity of phage buffer (PSB) to mites.

Following this, ten different bacteriophage infective against two MFB (*E. coli* and *S. aureus*) were investigated (Table 4.4) for their effects on mite survival and bacterial density. Mites were setup in chambers and administered purified high-titre bacteriophage lysates (Section 4.2.2.5) with approximately Log10 bacteriophage particles, mixed in a 1:1 ratio with lamb serum. Lamb serum (with added PSB) or water

only controls were also fed to mites. Survival and bacterial density were measured daily as previously described (Section 3.2.2.5).

Bacteriophage Intake by Mites

To measure intake of bacteriophage by mites following bacteriophage *in vitro* feeding experiment (Section 4.2.2.9), whole mites were crushed with ¼ strength Ringers solution as previously described (Section 3.2.2.5) and 10 µl of this whole-mite extract was assessed for bacteriophage presence using the spot-lysis assay (Section 4.2.2.7). Plates were incubated overnight at RT and ZOI (mm) was measured.

Table 4.4 Bacteriophage and respective bacterial hosts, used in mite feeding experiments.

Bacteriophage	Bacterial host
Ph1	*E.coli*
Ph2	
Ph3	
Ph4	
Ph5	
Ph6	
Ph8	
Ph9	
Ph10	*S.aureus*
Ph14	

4.2.2.10 Statistics

Principal component analysis (PCA) was used to analyse responses of bacteriophage lysates to chemical, temperature and pH treatments. Categorical results (+, <+, -, L) were transformed to numerical values (Table 4.5). PCA was analysed using GenStat v11 (VSN International Ltd, UK) with the individual chemical/enzymatic/temperature treatments as each component.

Effects of bacteriophage on MFB in liquid culture was analysed a using Kruskal-Wallis test in Genstat (v11.1). Effects of bacteriophage on survival were analysed using a General linear mixed model (GLMM), calculated in Genstat (v11.1) except Probit analysis which was calculated in MiniTab (v15) (MiniTab Inc.). Bacterial density values (CFU/mite) were transformed (Log_{10}+1) then analysed using a Linear mixed model (LMM) in Genstat v11.1).

Table 4.5 Transformation of categorical results of bacteriophage response to treatments for principal component analysis (PCA).

Categorical result	Effect of Treatment on Plaque Formation	Cluster analysis score
L	Bacteria lysed (not due to bacteriophage)	0
-	Plaques absent	0.5
<+	Plaques present-reduced compared to controls	5
+	Plaques present	10

4.3 Results

4.3.1 Isolation of MFB specific Bacteriophage from Environmental Samples

A range of environmental samples were screened for the presence of bacteriophage specific for MFB. Bacteriophage were isolated from 47% of environmental samples, which included eight water samples, three soil samples and five scab-infected fleece samples tested (Table 4.6). There were four environmental samples (W2, W3, W6, M7F) from which several different bacteriophage plaque morphologies were observed and these were selected as potentially different bacteriophage isolates. For example, sample W2, a water sample from Woodhouselee, contained bacteriophage specific to *E. coli* (G25) MFB (Figure 4.1), from which five isolates were purified (Table 4.6). In total 16 bacteriophage were isolated against MFB, ten infective for *E. coli* (G25), three infective for *S. aureus* (G16) and three infective for *A. faecalis* (G23). Lysogenic bacteriophage were isolated infective against *M. luteus* MFB (G19 and G29), however, this form of bacteriophage is unsuitable for bacteriophage therapy so these isolates were not included in this study

Figure 4.1 Clearances in *E. coli* (G25) bacterial lawn indicating lytic bacteriophage plaques present in water sample W2 infective against *E. coli* MFB. Five different plaques were selected based on different plaque morphologies. Scale bar: 10 mm.

4.3.2 Characteristics of MFB specific Bacteriophage

4.3.2.1 Plaque Morphology of MFB specific Bacteriophage

E. coli Bacteriophage

Isolated *E. coli* bacteriophage produced a range of plaque sizes and morphologies, ranging from small (1 mm) to large (6-7 mm) with defined edges or clear centre (4 mm) with a larger (8 mm) outer ring. All *E. coli* bacteriophage were isolated from environmental samples from the Edinburgh area (Woodhouselee or Easter Bush Farms) (Table 4.6).

S. aureus Bacteriophage

All the bacteriophage specific for *S. aureus* (G16) produced small (1-2 mm) plaques with clear defined edges (Table 4.6). Ph10 and Ph11 were isolated from scab-infected fleece (S39) originally from St Boswells, Scottish Borders and Ph14 was isolated from scab-infected fleece (S40) from Janetstown, Thurso respectively. Both scab-infected fleece samples were received in July 2009.

A. faecalis Bacteriophage

Alcaligenes faecalis-specific bacteriophage all had clear plaques with three plaque sizes observed (2, 4, 5 mm) (Table 4.6). Ph15 was isolated from sheep pen soil (D1, Aberdeenshire) and Ph16 and Ph17 were isolated from scab-infected fleece originally from an *in vivo* sheep scab culture (M7F, Moredun, Edinburgh).

4.3.2.2 Host range of MFB specific Bacteriophage

All isolated bacteriophage were tested for their ability to infect other MFB using the spot lysis assay. All bacteriophage isolated were only infective against the original isolation-bacterium indicating a high level of specificity in all bacteriophage.

Table 4.6 Summary of bacteriophage isolated from environmental samples infective against *E. coli, S. aureus* and *A. faecalis* MFB. Plaque diameter, morphology and phage reference number for each bacteriophage isolated is given.

Bacterial Host	MFB	Label	Origin of Environmental Sample	Plaque diameter (mm)	Plaque Morphology	Phage Reference Number
E. coli	G25	W1 1.1	Mud (water hole) Woodhouselee	8 / 4	clear centre with outer ring	**Ph1**
	G25	W2 1.1	Water (water hole) Woodhouselee	6-7	clear	**Ph2**
	G25	W2 2.1	Water (water hole) Woodhouselee	3	clear	**Ph3**
	G25	W2 3.1	Water (water hole) Woodhouselee	1	clear	**Ph7**
	G25	W2 4.1	Water (water hole) Woodhouselee	2-3	clear, defined edge	**Ph4**
	G25	W2 5.1	Water (water hole) Woodhouselee	1	clear, diffuse edge	**Ph8**
	G25	W3 1.1	Water from trough Woodhouselee	8 / 4	clear centre with outer ring	**Ph5**
	G25	W3 2.1	Water from trough Woodhouselee	1	clear, defined edge	**Ph6**
	G25	W4 1.1	Sheep faeces Woodhouselee	6	irregular, slightly diffuse edge	**Ph9**
	G25	W11 1.1	Mud from wood at Easter Bush-(wet)	1	clear	**Ph13**
S. aureus	G16	S39 1.1	Scab-infected sheep fleece (S39), St Boswells	2	clear, defined edge	**Ph10**
	G16	S39 2.1	Scab-infected sheep fleece (S39), St Boswells	1	clear, defined edge	**Ph11**
	G16	S40 1.1	Scab-infected sheep fleece (S40), Thurso	2	clear, defined edge	**Ph14**
A. faecalis	G23	D1 1.1	Sheep pen soil-Aberdeenshire	5	clear	**Ph15**
	G23	M7F 1.1	Scab-infected sheep fleece (M7), Edinburgh	2	clear	**Ph16**
	G23	M7F 1.2	Scab-infected sheep fleece (M7), Edinburgh	4	clear	**Ph17**

4.3.2.3 TEM of MFB-specific Bacteriophage

A series of measurements from transmission electron micrographs were taken using ImageJ (v 1.43) (NIH Image) and used for identification of bacteriophage isolates. Three bacteriophage isolates were used: Ph9, Ph11, Ph15, which infected *E. coli, S. aureus* and *A. faecalis* MFB respectively. Six measurements were taken: tail length, head length, head width, head side lengths (x 6), head area. The TEM results were used to classify isolated bacteriophage to a family/order level (Ackermann & Abedon, 2001; Ackermann, 2009).

E. coli Bacteriophage

Ph9 had an average head diameter of 47.5 nm ± 8.9E-4, average head length of 57.6 nm ± 4.55E-4 and average tail length of 140 nm ±2.85E-3. This bacteriophage was classified to be *Caudovirales, Siphoniviridae* due to its icosahedral head and tail measurements (Figure 4.2).

S. aureus Bacteriophage

Although repeated a number of times, with different bacteriophage lysate preparations, a suitable quality image for accurate measurements was not able to be achieved for Ph11.

A. faecalis Bacteriophage

Ph15 had an average head diameter of 42 nm ± 1.1, average head length of 45 nm ± 0.7 and average tail length of 131 nm ± 5.1. This bacteriophage had a striated tail (Figure 4.3) and was classified as an enterobacter bacteriophage within order *Caudovirales,* family *Siphoniviridae.*

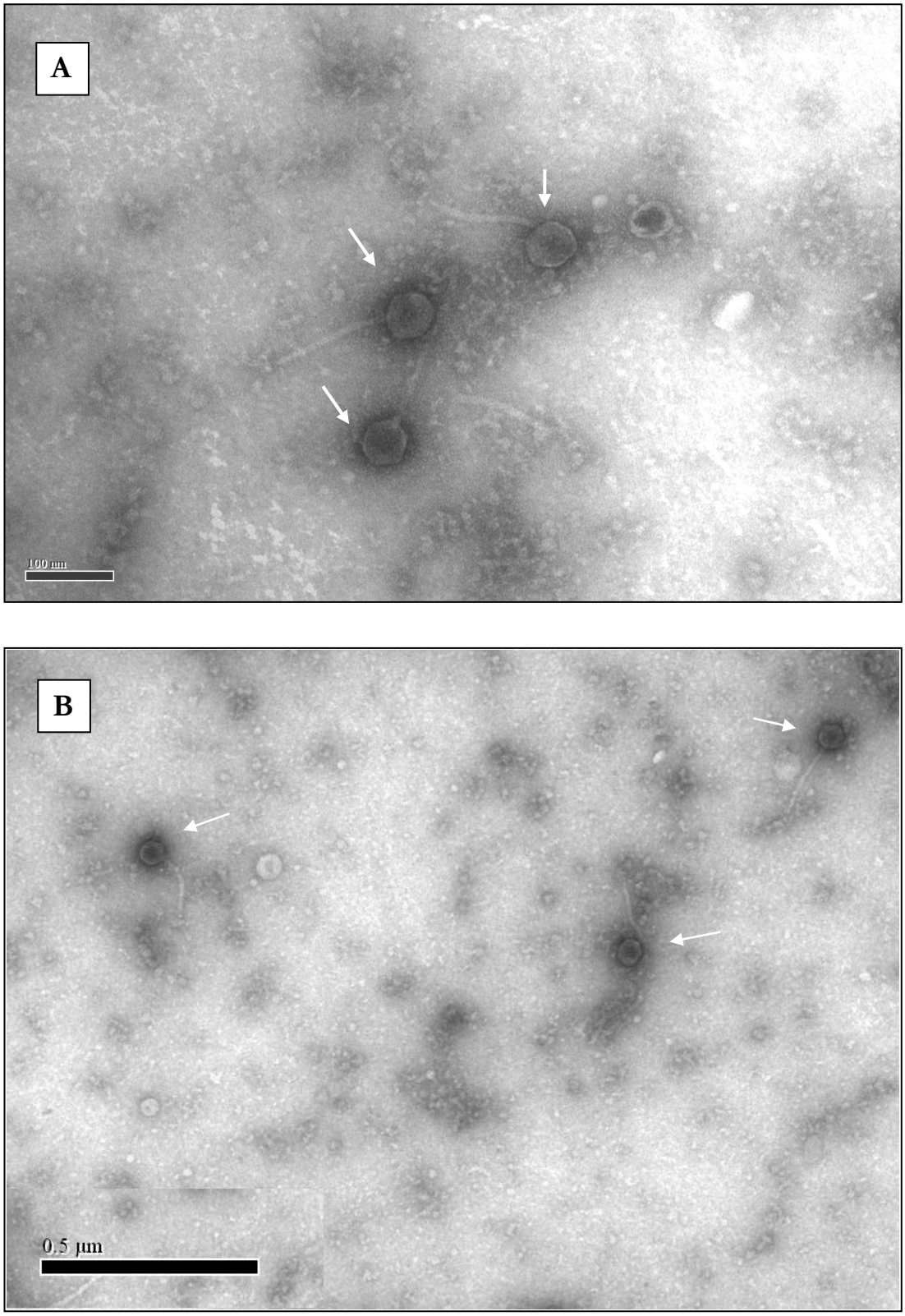

Figure 4.2 Transmission electron micrograph of *E. coli* bacteriophage (Ph9). Scale bar (**A**) 100 nm, (**B**) 0.5 µm.

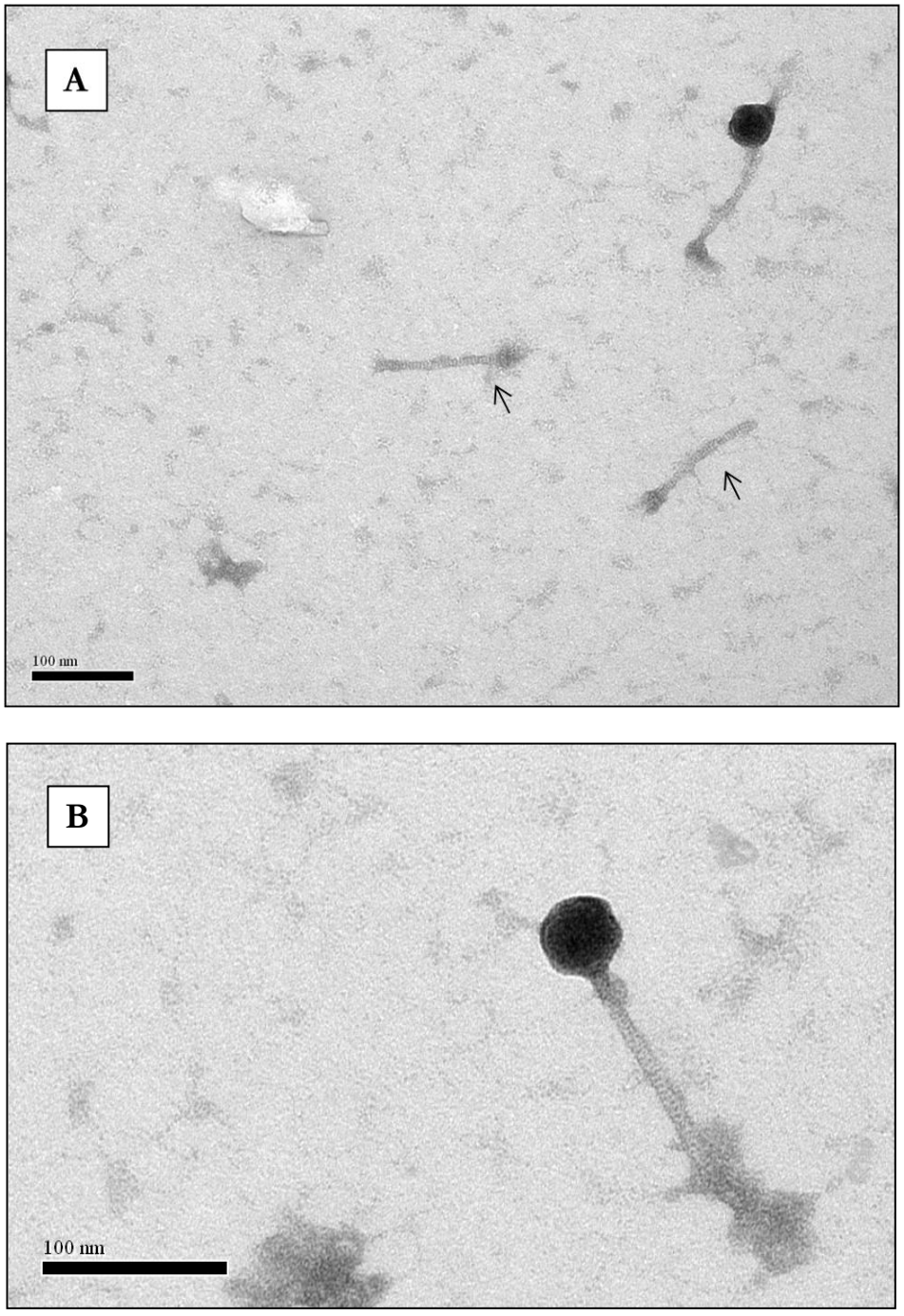

Figure 4.3 Transmission electron micrograph of *A. faecalis* bacteriophage (Ph15). (**A**) Arrows show two tails lacking head structures, (B) Bacteriophage with attached tail. Scale bar = 100 nm.

4.3.2.4 Restriction Enzyme Digests of MFB-specific Bacteriophage DNA

Lambda control

Lambda (λ) DNA was used as a control for restriction enzyme digests. Λ DNA was successfully digested with *HinfI*, *AluI* and *TaqI* (Figure 4.4). Therefore these three successful enzymes were used for digests of MFB-specific bacteriophage

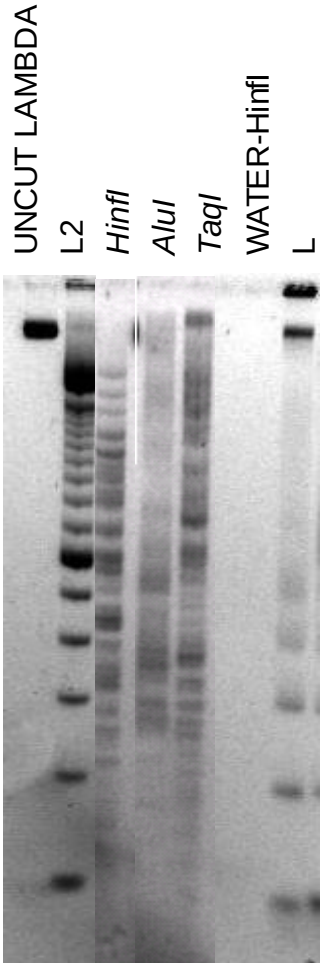

Figure 4.4 Restriction digests of Lambda (λ) DNA control. Ladders for size calibration: L = 1Kb^{+}, L2 = 100bp (Invitrogen). UNCUT = Extracted lambda DNA. Restriction enzymes::*HinfI, AluI*, *TaqI.*

E. coli Bacteriophage

DNA extraction produced good quantities and purities of DNA (>100 ng/μl) which was also visualised on an agarose gel (data not shown).

DNA from all ten *E. coli* bacteriophage (Ph1-9, 13) were first standardised to 20 μg/ml then digested with three different restriction enzymes to compare profiles, with varying success (Figure 4.5, 4.6). Ph7 produced blurred profiles for all three enzymes whereas others did not produce any bands (Ph4, Ph8). For the isolates that were successfully digested it was clear that Ph1, Ph2, Ph3, Ph4, Ph5 and Ph6 had very similar profiles for *HinfI* and *AluI* (Figure 4. 5A), in addition to similarities between Ph9 and Ph13 (Figure 4. 5B). This could indicate that Ph1-Ph6 are similar bacteriophage. The low resolution of bands visualised using the Bioanalyser did not allow for the production of dendrograms for *E. coli* bacteriophage.

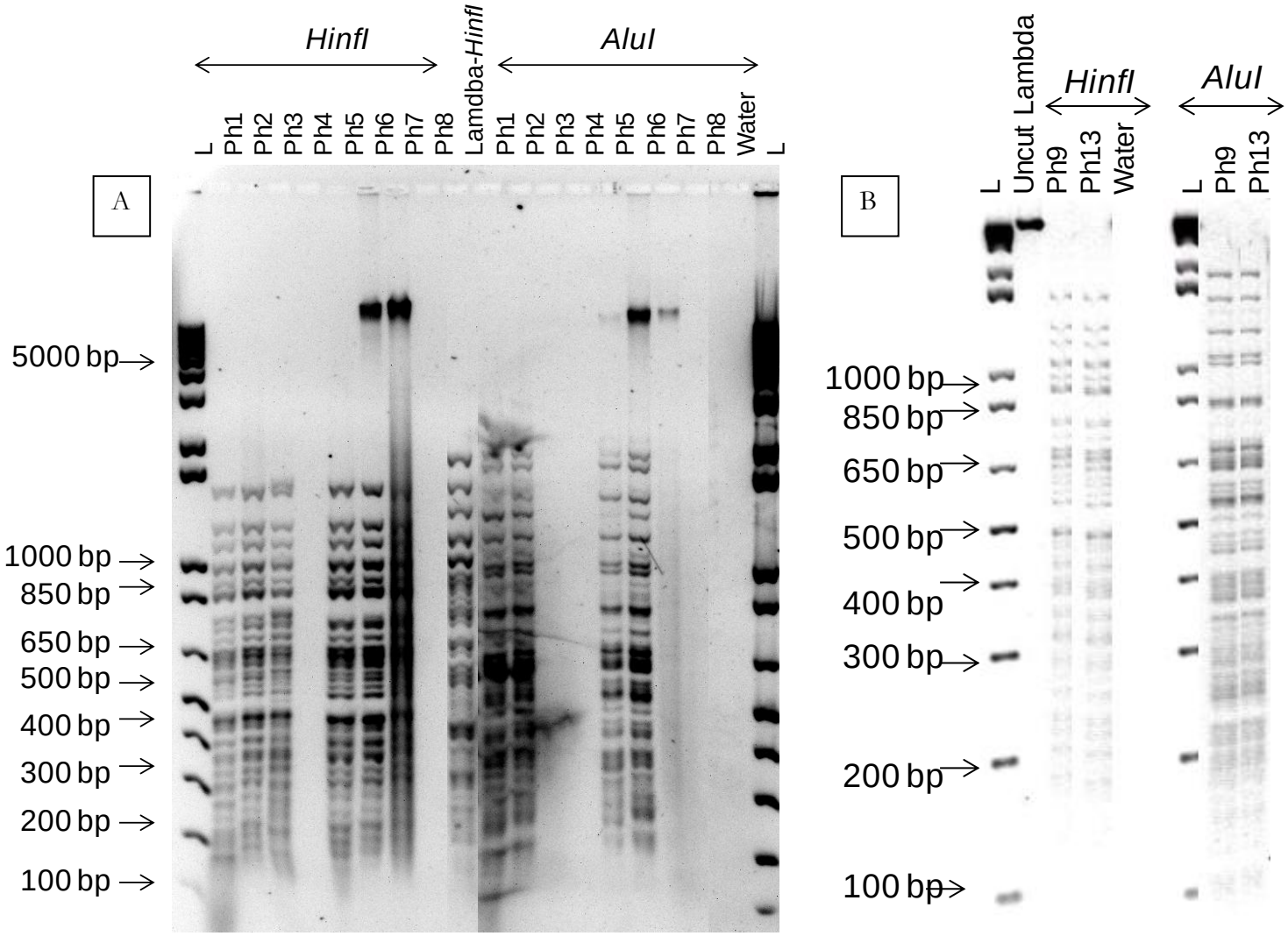

Figure 4.5 Restriction digest of *E. coli* bacteriophage (A:Ph1, Ph2, Ph3, Ph4, Ph5, Ph6, Ph7, Ph8, Ph9, Ph13) with *HinfI* and *AluI* restriction enzymes. (B) Ph9, Ph13 with *HinfI* and *ALuI*.Ladders for size calibration: L = 1Kb^{+}(Invitrogen).

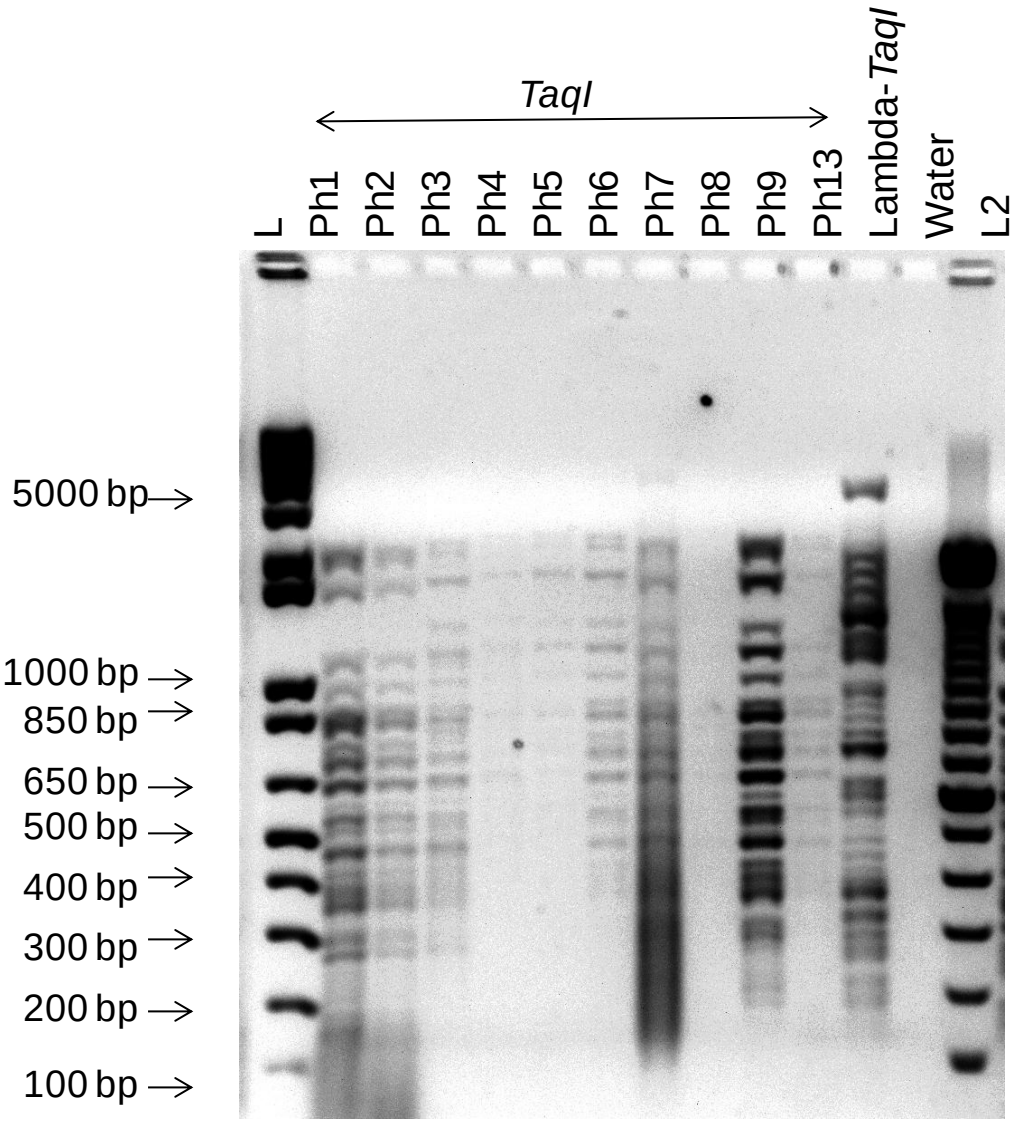

Figure 4.6 Restriction digest of *E. coli* bacteriophage (Ph1, Ph2, Ph3, Ph4, Ph5, Ph6, Ph7, Ph8, Ph9, Ph13) and lambda-DNA with *TaqI* restriction enzyme. Ladders for size calibration: L = 1Kb^{+}, L2 = 100 bp (Invitrogen).

S. aureus Bacteriophage

S. aureus bacteriophage provided the most challenges for DNA extraction, with low amounts (<50 ng/µl) continually produced following repeated DNA extractions for all three bacteriophage lysates (Ph10, 11, 14) for multiple lysate preparations. The bacteriophage genomic DNA was digested with three different restriction enzymes (*HinfI*, *AluI*, *TaqI*), however, all reactions except the lambda control failed with *S. aureus* bacteriophage DNA (data not shown). One potential reason could have been

insufficient bacteriophage DNA. Ten ml of bacteriophage lysate (mean Log9 PFU/ml) was originally used for each *S. aureus* bacteriophage DNA extraction, which is comparable to the other bacteriophage lysates used, yet even pooling DNA samples did not produce DNA levels for successful reactions.

A. faecalis Bacteriophage

A. faecalis bacteriophage lysates (Ph15, 16, 17) produced high levels of extracted DNA (>300 ng/μl) which were visualised on an agarose gel (data not shown). All three *A. faecalis* bacteriophage DNA were standardised to 20 μg/ml and successfully digested with *HinfI, AluI* and *TaqI* (Figure 4.7). Banding patterns produced were complex yet the restriction digest profiles of Ph15 and Ph17 were very similar for *HinfI* and *AluI* (Figure 4.7). This indicates that Ph15 and 17 could be grouped as similar bacteriophage. It was not possible to compare total number of bands due to smearing observed towards the bottom of the gel.

The products of RE digests were also run on an Agilent Bioanalyzer (Agilent Technologies), to produce a dendrogram for the *A. faecalis* bacteriophage (Ph15, 16 and 17) which indicated a close similarity of Ph15 and Ph17 (Figure 4.8). This technique can aid differentiation and selection of bacteriophage for testing the effect on MFB in liquid culture (Section 4.3.2.7).

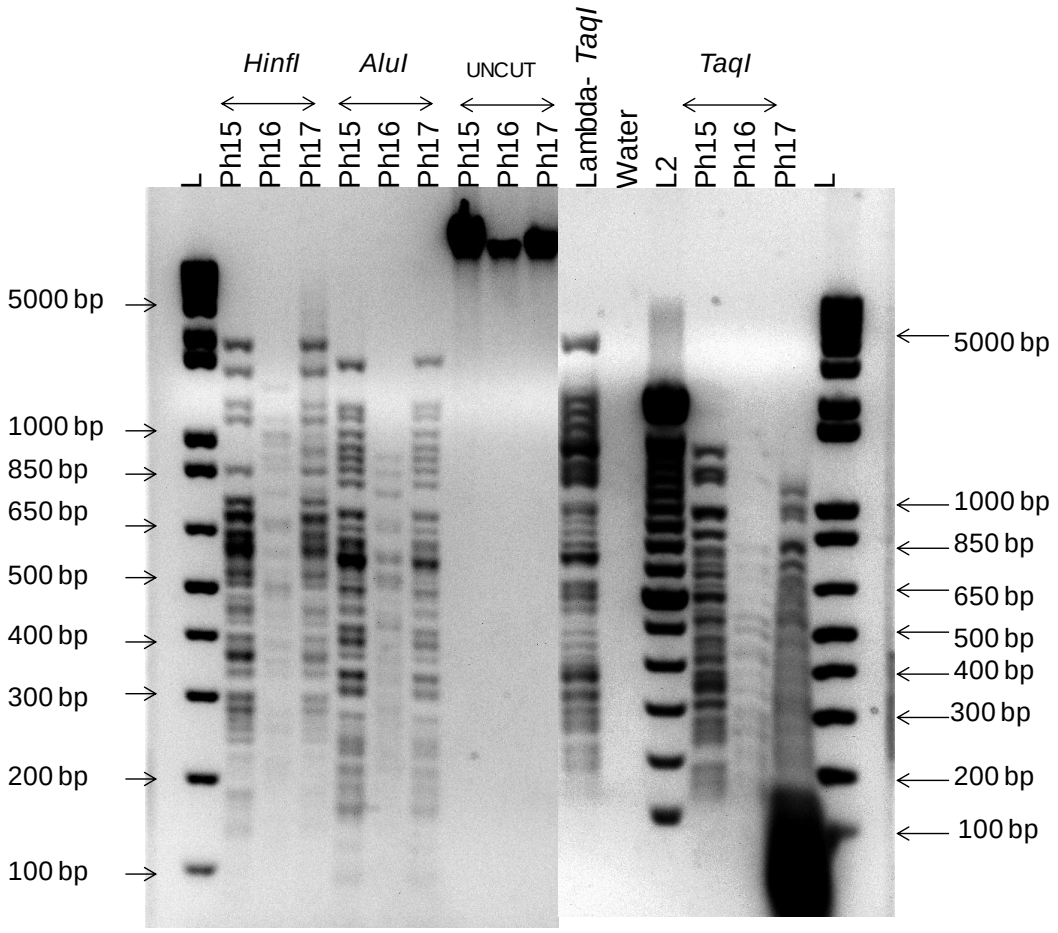

Figure 4.7 Restriction digests of *A. faecalis* bacteriophage (Ph 15, Ph16, Ph17). Ladders for size calibration L = 1Kb^{+}, L2 = 100bp (Invitrogen). Bacteriophage DNA digested with *HinfI, ALuI* and *TaqI* restriction enzymes and Lambda (*TaqI*) and water and Uncut DNA controls. Similar profiles were seen with Ph15 and Ph17,which indicates closely related groups.

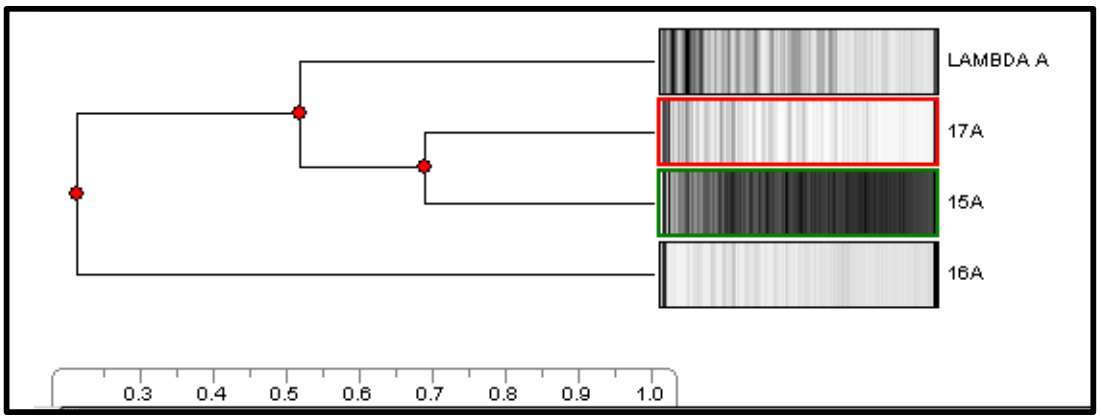

Figure 4.8 Dendrogram of bacteriophage restriction enzyme digest profiles of *A. faecalis* (G23) bacteriophage (Ph15, 16 & 17). Digested with *AluI*, compared to Lamda DNA digested with *AluI* (A). Scale shows Jaccard's similarity coefficient.

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4.3.2.5 Effect of Chemical, Enzyme and Temperature Treatments on MFB Plaque Formation

Effect of Treatments on MFB Controls

Chemical treatments were initially tested on MFB to observe any negative effects on bacterial growth as restriction of bacterial growth would subsequently effect the growth of bacteriophage.

Phage buffer adjusted to pH 12 inhibited growth of *E. coli* and *A. faecalis* but not *S. aureus*. EDTA inhibited growth of all three MFB (*S. aureus, A. faecalis, E. coli*), whereas SDS (1%) only inhibited growth of *S. aureus* and chloroform, ethanol (70%) and isopropanol only inhibited *A. faecalis* (Table 4.7). None of the enzyme treatments had a negative affect on MFB growth.

Table 4.7 Effect of pH-adjusted phage buffer (PSB), chemical and enzyme treatments on MFB growth. No effect of treatment on bacterial growth indicated by blank, bacterial growth completely inhibited by treatment (**X**). Each test was carried out in duplicate.

		Host Bacteria		
	Treatment	*E. coli* (G25)	*S. aureus* (G16)	*A. faecalis* (G23)
pH	pH 2			
	pH 3			
	pH 4			
	pH 5			
	pH 6			
	pH 7			
	pH 8			
	pH 9			
	pH 10.5			
	pH 12	**X**		**X**
Chemicals	Chloroform			**X**
	SDS (1%)		**X**	
	Triton (1%)			
	EDTA (0.01M)	**X**	**X**	**X**
	EtOH (70%)			**X**
	Isopropanol			**X**
Enzymes	Trypsin (10 mg/ml)			
	Lysozyme (1 mg/ml)			
	Ribonuclease A (1 mg/ml)			

Effects on *E. coli* Bacteriophage

All *E. coli* bacteriophage, except Ph1, Ph4 and Ph5, did not produce plaques with pH 2-adjusted phage buffer. They were able to produce plaques (except Ph3) with pH 3 adjusted-phage buffer (Table 4.8). Buffer adjusted to pH 12 did not support the growth of *E. coli* so this could not be tested directly on the bacteriophage lysates (Table 4.8). There were a range of responses to chemicals by *E. coli* bacteriophage. Ph3, Ph4, Ph8 were not inhibited by any of the chemical treatments, whereas Ph1 and Ph9 were inhibited by 4 and 5 treatments respectively (Table 4.9). *E. coli* bacteriophage were overall more resistant than the other bacteriophage (*S. aureus* or *A. faecalis*) to enzyme treatments with seven lysates resistant to them all. Ph1 and Ph9 were inhibited by three treatments each, and Ph13 was only inhibited by Proteinase K. All except Ph1 produced plaques after freeze/thaw treatment, however, all were inhibited or had restricted plaque formation after exposure to 60°C (Table 4.10).

Effects on *S. aureus* Bacteriophage

All three bacteriophage (Ph10, 11, 14) produced plaques in buffer adjusted to pH 3-10.5 yet were inhibited by pH 2 and pH 12 adjusted-buffer (Table 4.8). Each of the three *S. aureus* bacteriophage was affected by the chemical treatments in a different manner (Table 4.9). Ph10 and P14 were still able to produce lytic plaques after exposure to Triton (1%) and chloroform, whereas Ph11 was able to produce lytic plaques under these conditions yet were reduced compared to controls. Ph10 and Ph14, however, did not produce plaques after exposure to ethanol, whereas Ph11 was able to. In contrast Ph11 and Ph14 produced plaques at reduced levels with isopropanol yet Ph10 was completely inhibited by this chemical. All three bacteriophage produced plaques after exposure to all enzymes except trypsin and lysozyme, which inhibited Ph10 and Ph11 respectively. Moreover, although all bacteriophage produced plaques after freeze/thaw cycles, they were all inhibited by exposure to 60°C (Table 4.10).

Effects on *A. faecalis* Bacteriophage

Similarly to *S. aureus* bacteriophage, *A. faecalis* bacteriophage produced plaques in buffer adjusted to pH 3-10.5. Only one bacteriophage (Ph15) was able to produce plaques in pH 2 buffer (Table 4.8). *A. faecalis* bacteriophage produced lytic plaques after exposure to triton (1%) and SDS, however, the other chemical treatments inhibited *A. faecalis* bacterial grow so these were not tested on the bacteriophage lysates (Table 4.9). All

bacteriophage successfully produced plaques after the temperature treatments (freeze/thaw and 60°C) yet they were all inhibited by all enzyme treatments (Table 4.10).

Table 4.8 Results of pH-adjusted phage buffer on production of lytic plaques by bacteriophage compared to controls (no treatment). Plaques present (**+**), plaques present- reduced compared to controls (**<+**), plaques absent (**-**), bacterial lysis not due to bacteriophage (**L**). Each test was carried out in duplicate.

Bacteriophage	Host Bacterium	2	3	4	5	6	7	8	9	10.5	12
1	*E. coli* (G25)	+	+	+	+	+	+	+	+	+	L
2		-	+	+	+	+	+	+	+	+	L
3		-	+	+	+	+	+	+	+	+	L
4		+	+	+	+	+	+	+	+	+	L
5		<+	+	+	+	+	+	+	+	+	L
6		-	+	+	+	+	-	+	+	+	L
7		-	+	+	+	+	+	+	+	+	L
8		-	+	+	+	+	+	+	+	+	L
9		-	+	+	+	+	+	+	+	+	L
13		-	-	<+	<+	<+	<+	<+	<+	<+	L
10	*S. aureus* (G16)	-	+	+	+	+	+	+	+	+	-
11		-	<+	<+	<+	<+	<+	<+	<+	<+	-
14		-	+	+	+	+	+	+	+	+	-
15	*A. faecalis* (G23)	+	+	+	+	+	+	+	+	+	L
16		-	+	+	+	+	+	+	+	+	L
17		-	+	+	+	+	+	+	+	+	L

Table 4.9 Results of chemical treatments on production of lytic plaques by bacteriophage compared to controls (no treatment). Plaques present (**+**), plaques present- reduced compared to controls (**<+**), plaques absent (**-**), bacterial lysis not due to bacteriophage (**L**). Each test was carried out in duplicate.

Bacteriophage	Host Bacterium	Triton (1%)	SDS (1%)	Chloroform	EtOH (70%)	Isopropanol	EDTA (0.01M)
1	*E. coli* (G25)	-	-	+	-	-	L
2		-	<+	+	-	-	L
3		+	+	+	<+	+	L
4		+	+	+	<+	+	L
5		+	<+	<+	-	<+	L
6		+	<+	-	<+	<+	L
7		+	+	-	-	<+	L
8		+	+	<+	<+	<+	L
9		-	-	-	-	-	L
13		<+	<+	-	-	-	L
10	*S. aureus* (G16)	+	L	+	-	-	L
11		<+	L	<+	<+	<+	L
14		+	L	+	-	<+	L
15	*A. faecalis* (G23)	+	+	L	L	L	L
16		+	+	L	L	L	L
17		+	+	L	L	L	L

Table 4.10 Results of enzyme and temperature treatments on production of lytic plaques by bacteriophage compared to controls (no treatment). Plaques present (**+**), plaques present- reduced compared to controls (**<+**), plaques absent (**-**), bacterial lysis not due to bacteriophage (**L**). Each test was carried out in duplicate.

		Enzyme treatments				Temperature treatments	
Bacteriophage	**Host Bacterium**	**Proteinase K (20 mg/ml)**	**Trypsin (10 mg/ml)**	**Lysozyme (1 mg/ml)**	**Ribonuclease A**	**60°C**	**Freeze/ thaw**
1	*E. coli* (G25)	<+	-	-	-	-	-
2		+	+	+	+	-	+
3		<+	+	+	+	<+	+
4		<+	+	+	+	<+	+
5		<+	+	+	+	-	+
6		<+	+	+	+	-	+
7		+	+	+	+	-	+
8		<+	+	+	+	<+	+
9		-	-	<+	-	-	<+
13		-	<+	<+	<+	-	<+
10	*S. aureus* (G16)	+	-	<+	+	-	+
11		<+	<+	-	<+	-	+
14		+	<+	<+	+	-	+
15	*A. faecalis* (G23)	-	-	-	-	+	+
16		<+	-	-	-	+	+
17		-	-	-	-	+	+

4.3.2.6 Principal Component Analysis of MFB-specific Bacteriophage Profiles

PCA was used to differentiate bacteriophage isolates by analysing the effect of specific chemicals, enzymes and temperature (Section 4.3.2.5) on plaque formation. The recorded responses (plaque formation, reduced plaque formation, no plaque formation, or lysis not due to bacteriophage) were used to differentiate the bacteriophage isolates.

When analysing the responses of all bacteriophage together three main clusters were observed (Figure 4.9), divided by Pcscore_1 and 2. Together these clusters explained a total of 72.17% of the variation seen. Pcscore_1 (51.34%) was mainly composed of the responses to trypsin, triton-x and ribonuclease A, whereas pcscore_2 (20.83%) was composed of responses to pH 2-adjusted buffer and chloroform (Figure 4.9).

The bacteriophage present are infective against three MFB (*A. faecalis, S. aureus* and *E. coli*) and differences by host bacterium can be seen. All *A. faecalis* bacteriophage appeared very similar and clustered together (Figure 4.9A). *E. coli* bacteriophage, however, split into two distinct clusters (Figure 4.9C). Two *S. aureus* bacteriophage (Ph10 and Ph14) clustered together with some *E. coli* bacteriophage (Figure 4.9B).

In addition to the biochemical differentiations summarised in the analysis, it also allowed the culmination of other relevant supporting data, such as environmental origin of bacteriophage and plaque morphology (Section 4.3.1; Table 4.6). For example, there appeared to be a correlation between environmental sample type and PCA profile for the *E. coli* bacteriophage. Bacteriophage that originated from solid environmental samples (Table 4.6), for example mud or faeces were found in a cluster to the right of the dashed line (Figure 4.9C). However, bacteriophage sampled from water sources (Table 4.6) clustered to the left side of this line (Figure 4.9C).

Moreover bacteriophage were initially isolated and purified based on phenotypic differences exhibited on double agar plates (Section 4.3.2.1). For example, two plaques (Ph10 and Ph11) were picked from environmental sample W6 (scab-infected fleece S39). These two do not cluster together in this analysis indicating these may be different bacteriophage species/strains. Although bacteriophage isolates need to be sequenced for accurate taxonomic identification this analysis allows bacteriophage to be categorised by their clustering profiles.

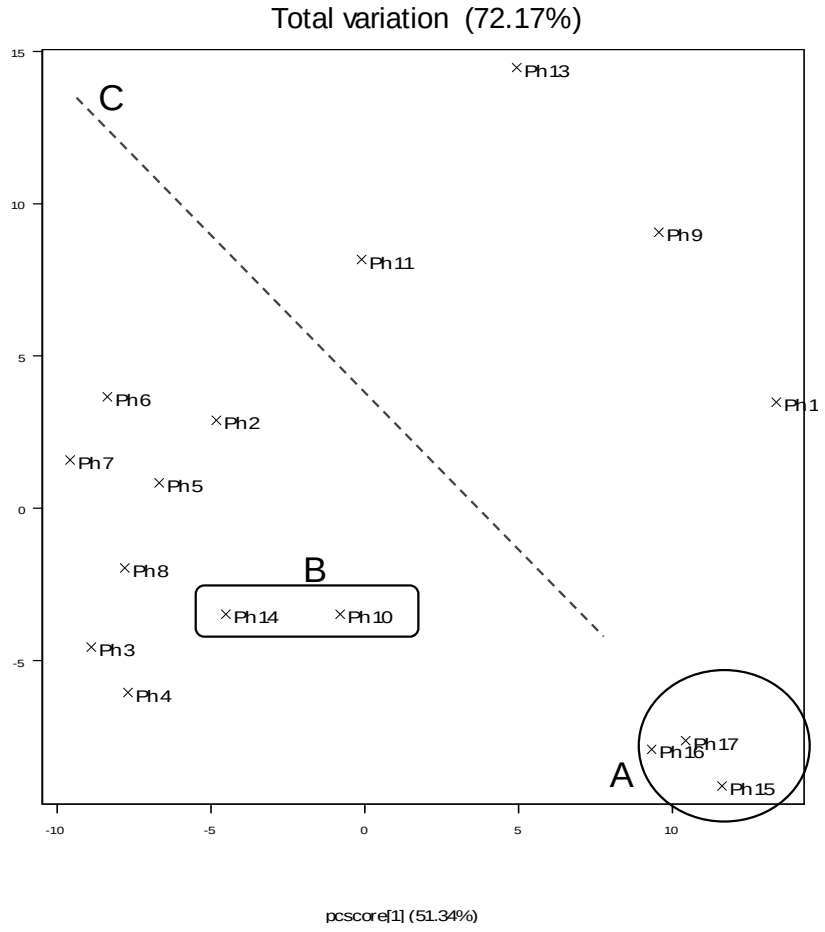

Figure 4.9 PCA of isolated bacteriophage based on responses to chemical, enzyme and temperature treatments. Explains a total of 72.17% of variation. Variation explained by pcscore 1 (51.34%) and pscore 2 (20.83%). Cluster of bacteriophage infective against MFB *A. faecalis* (G23; A), *S. aureus* (G16) clustered within *E. coli* bacteriophage (G25) cluster (B), dashed line (C) shows division of *E. coli* (G25) bacteriophage.

4.3.2.7 Effect of Bacteriophage on MFB in Liquid Culture

Each mite faecal bacteria was mixed with isolated bacteriophage at different ratios (MOI) in a liquid culture to observe affect on bacterial growth to assess potential for bacterial control (Park *et al.*, 2000). Each was compared to a LB only control and bacteria only positive control.

Initially a standard curve of optical density (OD) *versus* $\log_{10}$ transformed CFU/ml values was produced for the three MFB (*S. aureus, A. faecalis, E. coli*) for which lytic bacteriophage had been isolated (Appendix 9).

Effect of *S. aureus* bacteriophage

Initially five different MOIs (0.5, 1, 10, 20 and 50) of *S. aureus* bacteriophage Ph10 were added to a liquid culture of *S. aureus* MFB to observe the effects on bacterial growth. Ph10 decreased the growth of the *S. aureus* host compared to the control (Figure 4.10A). Ph10, at all MOI, resulted in a reduction, although not significant, in *S. aureus* host growth over the experimental time affected by bacteriophage treatment (H_6=10.46, P=0.107). As there was little difference between the effect of *S. aureus* bacteriophage at a MOI ratio of 0.5 compared to 50, subsequent experiments used two MOI ratios only (0.5 and 1).

The two other *S. aureus* bacteriophage (Ph11 and Ph14) were tested for their effects on *S. aureus* MFB growth in liquid culture. These two bacteriophage only had negative effects on growth of the *S. aureus* host after 6 h of incubation which reduced the density by 24 h (Figure 4.10B). There was no significant effect on treatment on *S. aureus* growth (H_6=10.98, P=0.203).

Also included in this experiment was a 'cocktail' of all *S. aureus* bacteriophage (Ph10, 11 and 14 MOI 0.5), which restricted *S. aureus* growth to half that of the bacterium-only control yet was less effective than the single bacteriophage lysate treatments.

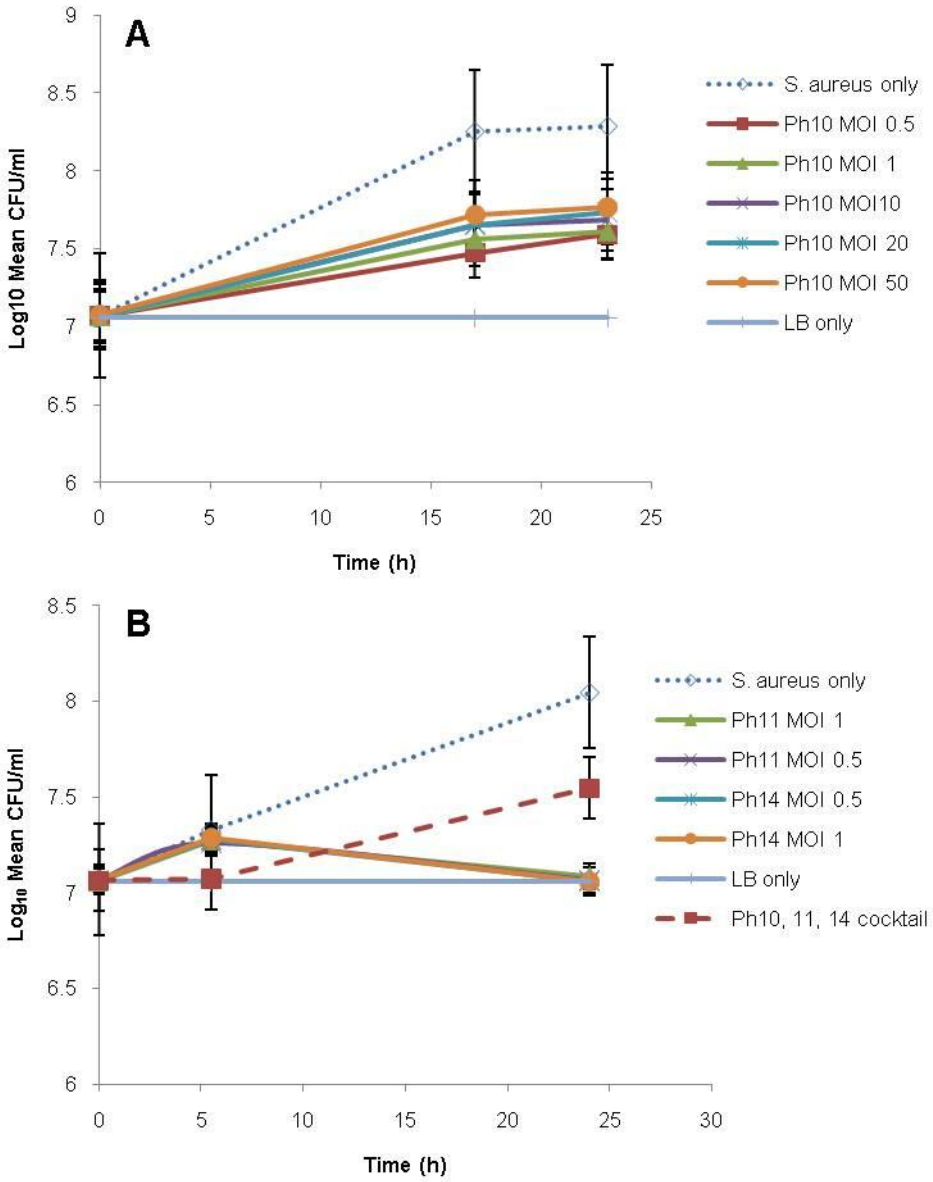

Figure 4.10 Effect of bacteriophage on growth of *S. aureus* MFB liquid culture. (**A**) Ph10: Dashed line represents control culture of *S. aureus* only, solid lines represent Ph10 at five different MOI (0.5, 1, 10, 20, 50). No significant effect of Ph10 dose (H_6=10.46, *P*=0.107). (**B**) Ph11, Ph14 at 0.5,1 MOI. Also a cocktail of Ph10, 11, 14 at MOI 0.5. No significant effect of treatment on *S. aureus* growth (H_6=10.98, *P*=0.203).

Effect of *A. faecalis* bacteriophage

Bacteriophage infective against *A. faecalis* MFB at MOI of 0.5 and 1 did not restrict growth on *A. faecalis* in liquid culture, with no significant effect of treatment on bacterial growth (H_7=10.49, P=0.162) (Figure 4.11).

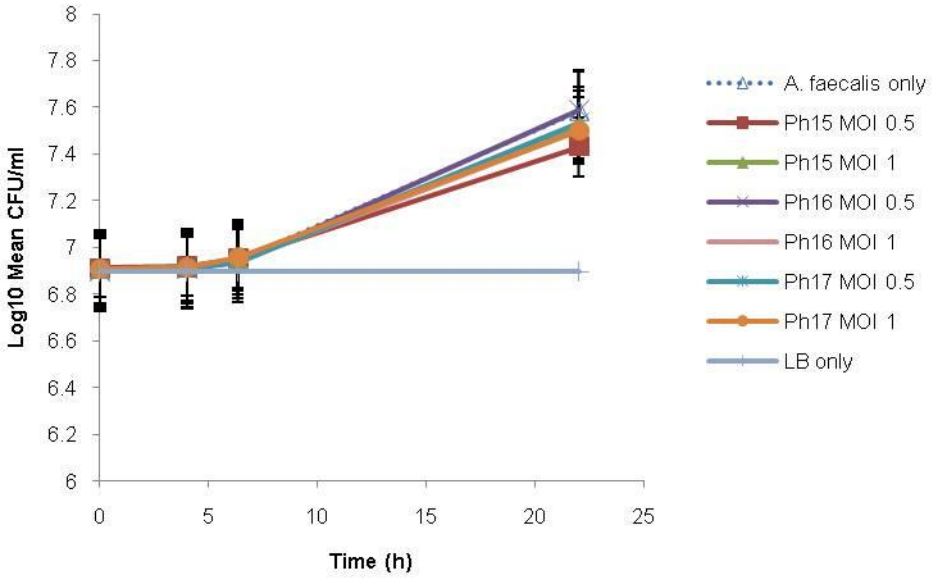

Figure 4.11 Effect of bacteriophage on growth of *A. faecalis* MFB in liquid culture. Dashed line represents control culture of *A. faecalis* (G23) only, solid lines represent Ph15, Ph16, Ph17 at MOI (0.5,1). No significant effect of treatment (H_7=10.49, P=0.162) on *A. faecalis* growth.

Effect of *E. coli* Bacteriophage

Four *E. coli* bacteriophage (Ph1, Ph4, Ph9 and Ph13) were added to *E. coli* MFB at a MOI of 0.5 or 1 in liquid culture. Growth of the bacteria was suppressed by all bacteriophage treatments until 6 h compared to control, after which *E. coli* grew steadily (Figure 4.12). Overall all bacteriophage treatments had a lower growth than bacteria only control. The *E. coli* bacteriophage cocktail did not have a stronger effect on bacterial growth than some of the single bacteriophage treatments. There was a significant effect of treatment on bacterial growth (H_{10}= 26.62, P = 0.003).

These experiments showed that bacteriophage dynamics varied in the time before restriction of bacterial growth. This is an important concept for bacteriophage biocontrol application.

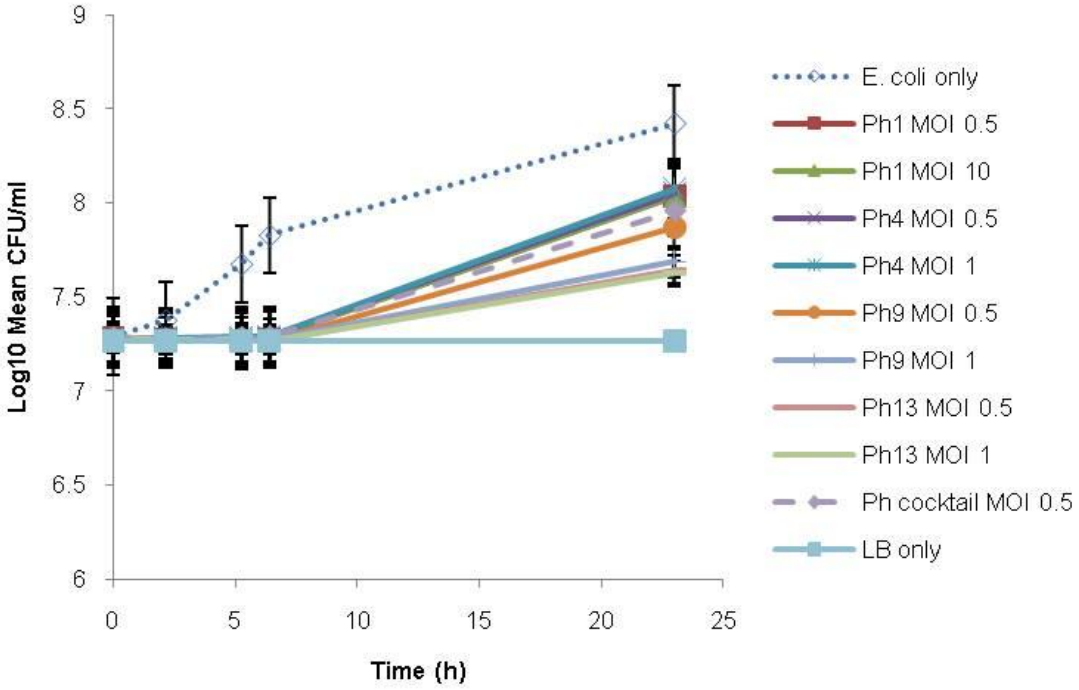

Figure 4.12 Effect of bacteriophage on *E. coli* MFB in liquid culture. Dashed line represents control culture of *E. coli* only, solid lines represent Ph1, 4, 9, 13 at MOI (0.5,1) and a cocktail (mix of all four bacteriophage). Significant effect of bacteriophage treatment (H_{10}= 26.62, $P = 0.003$) on *E. coli* growth.

4.3.3 *In Vitro* Bacteriophage Feeding Experiment

4.3.3.1 Bacteriophage Buffer Toxicity

P. ovis mites were fed bacteriophage buffer in lamb serum to assess the effect on *P. ovis* survival. When compared to lamb serum control, mite survival curves indicated that bacteriophage buffer alone did not have toxic effects on *P. ovis* mites (Figure 4.13). Moreover probit analysis of LT_{50} values showed no difference between treatments (Table 4.11). This indicates that the buffer alone does not have toxic effects on *P. ovis* survival.

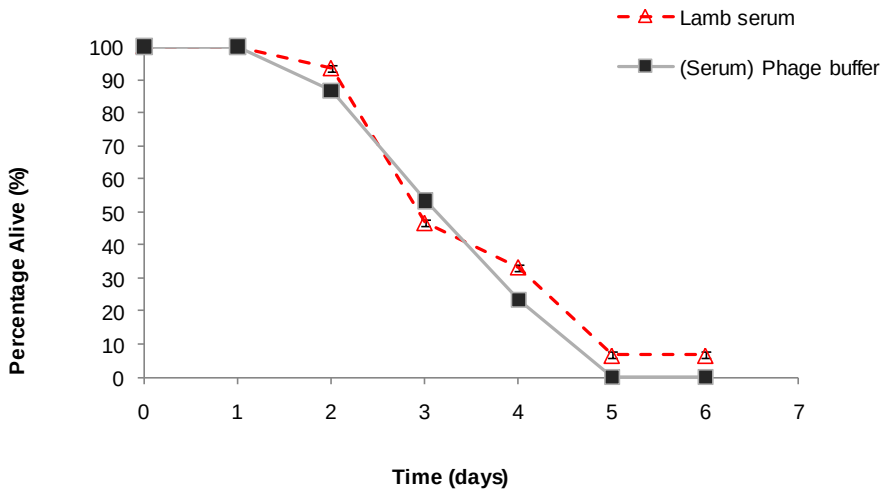

Figure 4.13 Effect of bacteriophage buffer on *P. ovis* survival. Survival of *P. ovis* mites (with LT_{50} in days) with lamb serum control (3.413) or bacteriophage buffer (PSB) dissolved in lamb serum (3.131). There was no significant difference between treatments. Number of mites at the start: Lamb serum (30), Bacteriophage buffer in serum (30).

Table 4.11 LT_{50} values for toxicity test of bacteriophage buffer (PSB in lamb serum) compared to lamb serum control. There was no significant difference between the two treatments, indicated by same letter after percentile value.

Treatment	Mites alive at start (0d)	Mites alive at end (6d)	LT_{50}	Standard Error	95% Fiducial CI	
					Lower	Upper
Lamb serum	**30**	**2**	3.413a	0.152	3.104	3.716
Bacteriophage buffer (PSB)	**30**	**0**	3.131a	0.1291	2.869	3.393

4.3.3.2 Effect of Bacteriophage on *P. ovis* Survival

P. ovis survival was recorded over five days to test the timing of any effect of bacteriophage on *P. ovis* mites. Four different bacteriophage were tested, two infective for *E. coli* MFB (Ph8, Ph9) and two infective for *S. aureus* MFB (Ph10, Ph14) owing to the results of PCA and the effect of bacteriophage lysates on MFB in liquid culture.

Within this time there were significant differences observed in mortality between treatments. When analysed with GLM, survival of mites was significantly affected by treatment ($F_{4,140}$=18.02, P<0.001), time sampled ($F_{1,140}$=503.6, P<0.001) and there was an interaction of treatment*time sampled ($F_{4,140}$=7.99, P<0.001).

Mites fed lamb serum (with phage buffer) only had the highest survival rates of all treatments (Figure 4.14) whereas mites responded to each bacteriophage treatment differently. Although Ph14 (*S. aureus* bacteriophage) appeared to have the most dramatic effect on mortality within the first 24 h, the rate of mortality declined. Ph8 (*E. coli* bacteriophage), however, resulted in the highest mortality with only 12% of the mites remaining alive on day five. Ph9 (*E. coli* bacteriophage) and Ph10 (*S. aureus* bacteriophage) appeared to produce similar mortality effects, with all bacteriophage treatments resulting in overall higher mortality than the lamb serum control.

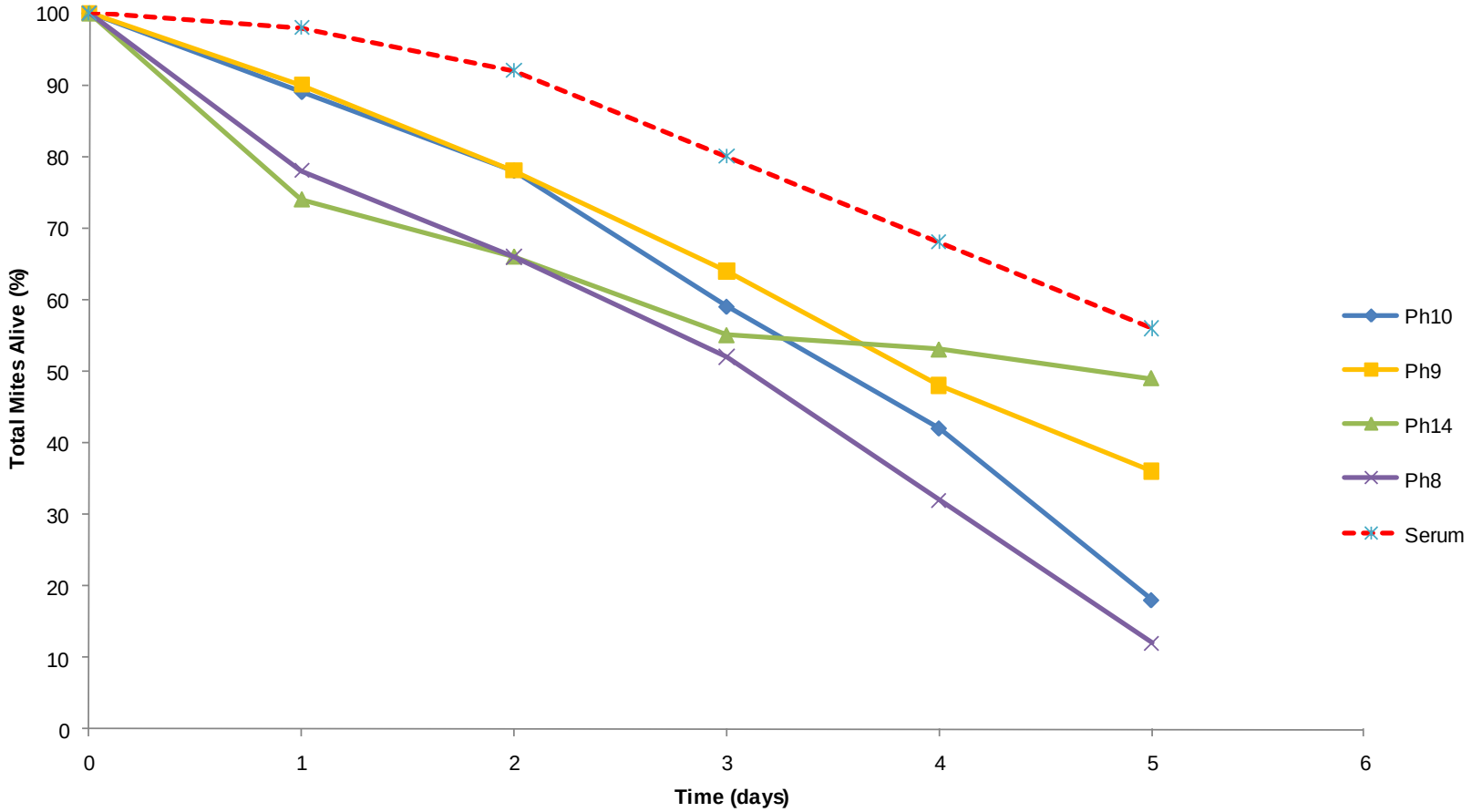

Figure 4.14 Effect of bacteriophage on *P. ovis* survival. Survival curves for *P. ovis* mites fed lamb serum or lamb serum with added bacteriophage (Ph8, 9, 10, 14) at a concentration of Log 10 PFU/ml. Ph8 and Ph9 are infective against *E. coli*, Ph10 and Ph14 are infective against *S. aureus*. Significant effects on mite survival of treatment ($F_{4,140}$=18.02, P<0.001), time sampled ($F_{1,140}$=503.6, P<0.001) and the treatment*time sampled interaction ($F_{4,140}$=7.99, P<0.001) was seen. Number of mites in each treatment at start = 100.

4.3.3.3 Effect of Bacteriophage on *P. ovis* Bacterial Density

Simultaneously to the effect of bacteriophage lysates on *P. ovis* survival (Section 4.3.3.2), the effect of bacteriophage on mite bacterial density was investigated with *P. ovis* mites fed one of four bacteriophage, two infective against *E. coli* (Ph8, Ph9) and two against *S. aureus* (Ph10 and Ph14).

Overall mites dead at time of sampling had a higher bacterial density than live mites after administered with the bacteriophage treatments with a significant effect of status (alive/dead) ($F_{1,51}$=6.29, P=0.015) observed (Figure 4.15).

There were a number of significant factors affecting the bacterial density of *P. ovis* including treatment ($F_{4,51}$=4.44, P=0.004), time ($F_{1,51}$=15.97, P<0.001), status (alive/dead; $F_{1,51}$=6.29, P=0.015) and treatment-time-status interaction ($F_{4,51}$=11.03, P<0.001) (Figure 4.15).

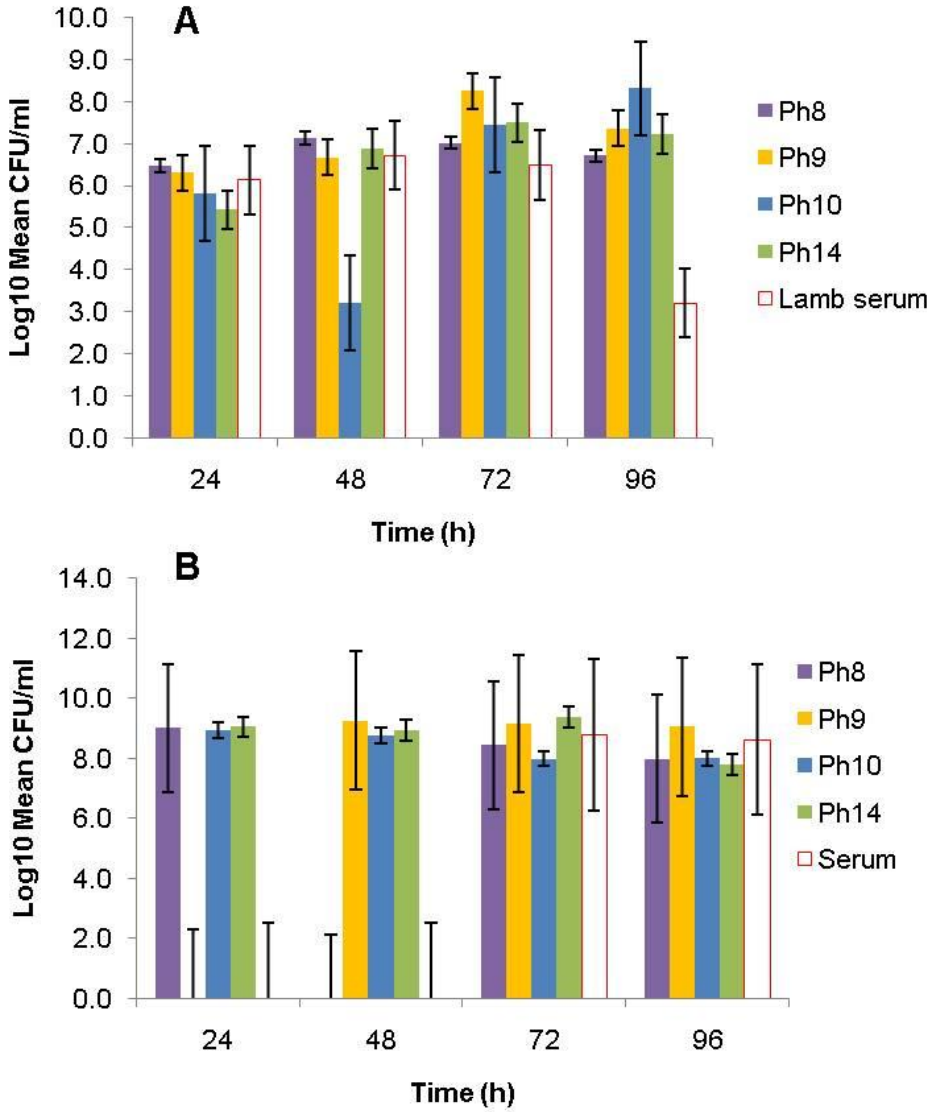

Figure 4.15 Mean bacterial density of *P. ovis* mites administered with bacteriophage treatments. Mites fed Ph8, Ph9 (infective for *E. coli* MFB) or Ph10, Ph14 (infective for *S. aureus* MFB) with Log10 PFU/ml starting concentration. (**A**) Alive mites and (**B**) Dead mites. Significant effects on bacterial density of treatment ($F_{4,51}$=4.44, *P*=0.004), time ($F_{1,51}$=15.97, *P*<0.001) and status (alive/dead) ($F_{1,51}$=6.29, *P*=0.015) was observed. N=10 per treatment.

Mites were administered six bacteriophage infective against *E. coli* (Ph1 –Ph6) in lamb serum to investigate effect on internal bacterial denisty. Over 86 h, mites were sampled, surface sterilised, crushed and plated onto agar plates. A decrease in bacterial density of live mites compared to lamb serum controls was observed for all bacteriophage treatments (Figure 4.16). There was a significant effect of both treatment (F_7=4.23, *P*=0.003) and time (F_4 =2537.18, *P*<0.001) on bacterial density in *P. ovis* mites. ZOI

(mm), as a measure of bacteriophage presence was recorded after *P. ovis* mites were fed *E. coli* bacteriophage (Ph1-6). Crushed mite extracts were spotted onto pre-inoculated agar and ZOI measured. A lot of variation was seen (Figure 4.17) yet three bacteriophage (Ph2, 5 and 6) increased over the course of the experiment. There was a significant affect of treatment (F_7=3.47, *P*=0.008) and time (F_4=4.32, *P*=0.008) for ZOI in this experiment.

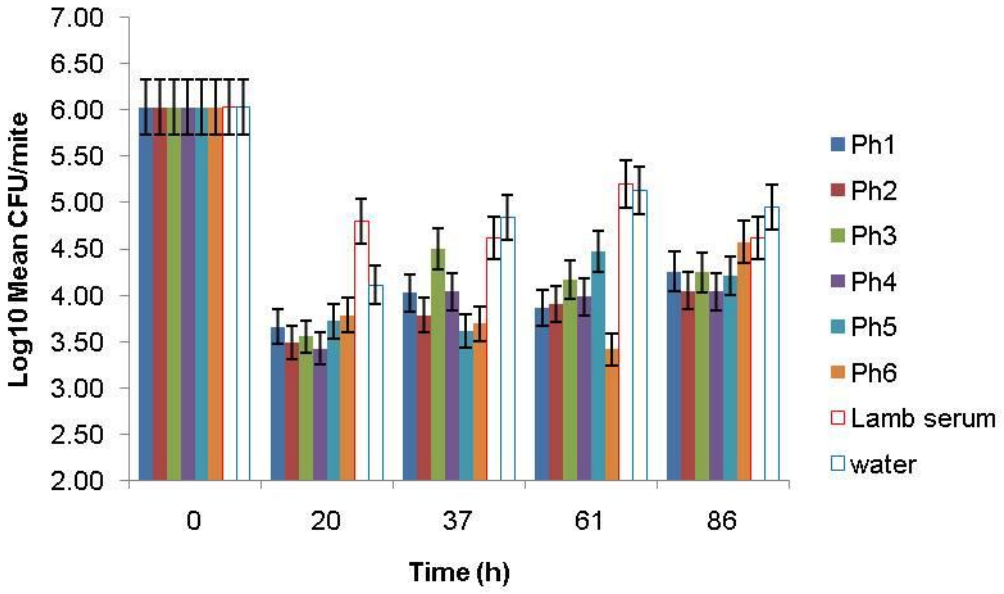

Figure 4.16 Bacterial density (CFU per mite) after administration of *E. coli* bacteriophage (Ph1-6). Bacteriophage dose of Log10 PFU/ml in serum compared to lamb serum positive control. Error bars are sem, N=3 per treatment. There was a significant effect of treatment (F_7=4.23, *P*=0.003) and time (F_4 =2537.18, *P*<0.001) on bacterial density in *P. ovis* mites.

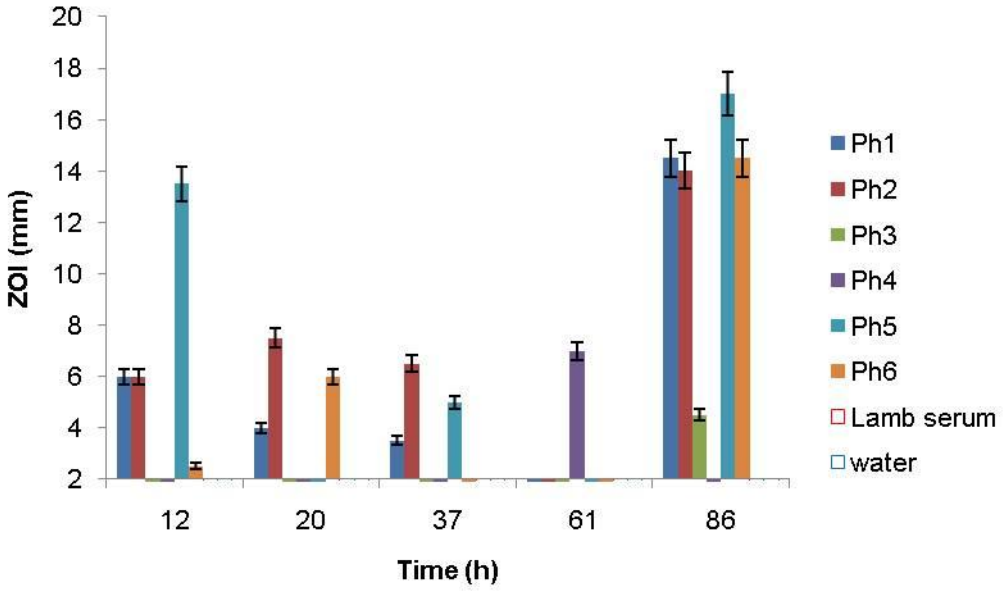

Figure 4.17 Zone of inhibition (mm) for crushed *P. ovis* mites after administration with *E. coli* bacteriophage (Ph1-6), lamb serum and water controls. Bacteriophage dose of Log10 starting PFU/ml concentration in lamb serum. Error bars are se, N=3 per treatment. There was a significant effect of treatment (F_7=3.47, *P*=0.008) and time (F_4=4.32, *P*=0.008) for plaque size.

Bacteriophage Recovery from *P. ovis* mites

To test whether bacteriophage were entering the internal cavities of the mite after administration in lamb serum, bacteriophage recovery was carried out by crushing surface sterilised mites that had previously been fed bacteriophage (Section 4.3.3.2) and plating onto an agar plate inoculated with the apprpriate host MFB. Mites were first tested for the presence of bacteriophage infective against *E. coli* or *S. aureus* MFB, yet none were isolated.

Mites were fed Ph8, Ph9 (*E. coli* bacteriophage) and Ph10, Ph14 (*S. aureus* bacteriophage) and tested daily for bacteriophage presence internally. Within 24 h until the end of the experiment, bacteriophage was detected from surface-sterilised crushed mites. The recovered bacteriophage were purified from plaques then characterised by plaque morphology and cross-reactivity with other MFB. All recovered bacteriophage had characteristics matching to the original isolate so were assumed to be the same as originally administered. Molecular sequencing of isolated bacteriophage, however, would be required to definitely ascertain and confirm bacteriophage species.

4.4 Bacteriophage Discussion

4.4.1 Isolation of Bacteriophage against Mite Faecal Bacteria

Bacteria previously isolated from *P. ovis* faecal trails (Chaper 2; Section 2.3.2) were used to isolate bacteriophage from environmental samples. There were a number of reasons for using these specific bacteria:

The mite faecal bacteria (MFB) were isolated through culture-dependent methods. This produced pure bacterial isolates against which environmental bacteriophage could be isolated through enrichment. Moreover, the bacteria had been identified by molecular DNA sequencing (Chapter 2; Table 2.7) which aided in bacteriophage identification. Other bacteria identified in *P. ovis* by culture-independent methods could in future also be cultured to isolate bacteriophage, if a suitable microbial target was identified.

This study has shown that it is possible to isolate bacteriophage from the environment which are infective against, and specific to MFB. Sixteen bacteriophage were isolated in total, with ten bacteriophage infective against *Escherichia coli* (G25) and three bacteriophage infective against *Staphylococcus aureus* (G16) and *Alcaligenes faecalis* (G23). Further sampling from a greater range of environments would be required to enable bacteriophage isolation that are infective against other MFB. Isolated bacteriophage were only infective against the bacterial species originally used for enrichment, therefore no cross-reactivity between bacterial species was observed. This specificity is valuable for bacteriophage biocontrol, yet further tests on a broader range of bacteria not associated with mites is required before applied use. Other bacterial species associated with sheep scab disease identified in Chapter 2 were not used for further cross-reactivity tests due to the lack of cultures as DNA molecular sequencing was used for identification. The three MFB that successfully isolated bacteriophage are fairly ubiquitous in the environment so may not be specific enough to *P. ovis* mites to be suitable bacteriophage biocontrol targets. For example, *E. coli* (G25) is present in a number of environments but has high levels of genetic diversity (Johnson *et al.*, 2004) and strain differences between habitats, which may include isolates from *P. ovis*. *E. coli* is a much studied and frequently used model bacterium, so for this reason it was utilised for initial bacteriophage experiments. Additionally, *E. coli* is the closest free-living relative to *Buchnera,* the obligate symbiont of aphids (Tamas *et al.*, 2002) which makes it an interesting target.

S. aureus, A. faecalis and *E. coli* (G16, G23, G25 respectively) have previously been isolated from sheep skin under both healthy and disease conditions (Meyer *et al.*, 2001; Dixon *et al.*, 2007) and could be potential causes of opportunistic infections associated with sheep scab disease (Bates, 2003). For this reason, bacteriophage against these MFB may be suitable as part of a 'cocktail' of therapeutic bacteriophage with the aim of treating the skin lesions, or in combination with more specific bacteriophage to target *P. ovis*-specific bacteria. Bacteriophage are found in the same environment as the bacteria they infect, however, bacteriophage were only isolated from environmental samples against three MFB in this chapter. This could indicate the other MFB are more closely associated with *P. ovis* than the wider environment.

The arthropod endosymbiont *Comamonas* sp. was identified in *P. ovis* mites by specific PCR assays in this study (Chapter 2; Section 2.3.3.5). Although identified by culture-independent methods, some species of this bacterium are culturable (Setala, 2000) and because of this it may be a potential target for bacteriophage biocontrol. Further work is required to isolate bacteriophage specific for *Comamonas* sp. as it was not possible in this study and to also understand its infection dynamics within the mite host.

Lysogenic bacteriophage were also isolated in this chapter, against two MFB (G19, G24), both identified as *Micrococcus luteus*. This type of bacteriophage is not suitable for bacteriophage biocontrol as virions are produced without destruction of the bacterial host (Abedon, 2008). Therefore no further analysis was carried out with these isolates.

The double layer technique used in this study is a straightforward and inexpensive method for rapid examination of a large number of environmental samples. It is also easy to collect purified bacteriophage from plaque assays as the solid nature of agar stops the virus progeny from moving too far away from the original host (Brock, 1970).

It must be remembered that the enrichment method used to isolate bacteriophage in this study is qualitative. The quantity of different bacteriophage in the original environmental samples is unknown. Future studies to investigate abundance could use epifluorescence microscopic counting (Wolf *et al.*, 2003).

4.4.2 Bacteriophage Characteristics

4.4.2.1 Transmission Electron Microscopy

Visualisation of bacteriophage lysates by transmission electron microscopy (TEM) was carried out to enable identification of bacteriophage to a family or order level (Clokie & Kropinski, 2009). Only three bacteriophage lysates (Ph9, Ph11, Ph15) each infective against a different MFB were photographed due to financial constraints. Although visualisation allowed characterisation such as presence of a tail, genetic sequencing would be required for identification to a species level. Ph11 could not be successfully optimised for TEM observation.

The *E. coli* and *A. faecalis* bacteriophage were both tailed, and classed within the S*iphoniviridae* family. These bacteriophage are frequently isolated and along with *Myoviridae* and *Podoviridae,* make up 96% of bacteriophage in culture (Wolf *et al.*, 2003).

4.4.2.2 DNA Extraction

DNA extraction was successful for the majority of bacteriophage isolates; however, the extraction method and resulting DNA quantity may be affected by the concentration of bacteriophage particles in the lysate and the genome sizes of the bacteriophage. For example, *E. coli* bacteriophage genomes can range in size from 3.5-168.9 kb, whereas *S. aureus* bacteriophage are much smaller about 41.4 kb (Kimball, 2010), this may be a reason why it was difficult to produce successful extractions from *S. aureus* bacteriophage. Future bacteriophage DNA extractions may be improved by the use of bacteriophage-specific DNA extraction kits, such as carboxyl functionalised magnetic nonporous microspheres (Kahankova *et al.*, 2009).

4.4.2.3 Restriction Enzyme Digests

The restriction enzyme profiles produced from bacteriophage can assist in the characterisation and differentiation of bacteriophage isolates. Restriction enzymes require double stranded DNA for their recognition sequence (Pelczar *et al.*, 1993). *S. aureus* (G16) bacteriophage were not successfully digested by any of the restriction enzymes. This may be as a result of the small quantity of DNA produced from extractions or it may indicate *S. aureus* (G16) bacteriophage isolated in this chapter do not have double stranded DNA or are even RNA bacteriophage. Future tests, such as the DNA type (i.e. DNA or RNA) which is important for restriction enzyme digests, could be determined by the diphenylamine test, in which the chemical reacts with

deoxyribose sugar if present, turning the solution blue (Steinberg *et al.*, 1976). Also the presence of double or single stranded DNA could be determined using the specific melting temperature (Steinberg *et al.*, 1976).

Two of the three *A. faecalis* bacteriophage isolates exhibited similar banding patterns after digestion with three restriction enzymes (*HinfI, AluI and TaqI*) and *E. coli* bacteriophage were clustered in two main groups which was also seen with the PCA profiles.

4.4.2.4 Bacteriophage Response to Chemicals, Enzymes and Temperature

Investigation into the resistance of bacteriophage to certain chemicals and enzymes is important to test their stability and suitability as bacteriophage biocontrol agents. Bacteriophage can be found in a number of extreme environments, such as thermal vents (Williamson *et al.*, 2008) or the arctic (Sawstrom *et al.*, 2007), suggesting bacteriophage can adapt to and withstand harsh conditions.

The chemicals chosen in this chapter are frequently used to test bacteriophage (Rigby *et al.*, 1989), however, they lacked conditions that may be encountered by the bacteriophage if applied to sheep as a scab control method, such as organic fleece components. The tests for chemical stability are important, however, as they do potentially mimic some factors in association with application on sheep, such as encountering the stomach acid of sheep if bacteriophage are ingested, as most bacteriophage cannot grow in acidic environments (Dabrowska *et al.*, 2005), or elevated temperature, as skin will increase in temperature with inflammation of the scab lesion, due to an increase in blood cells (Merck & Co., 2010). In addition to the characterisation of bacteriophage, chemicals are often used to find chemicals with negative effects on bacteriophage. Ebrecht *et al.,* (2010) investigated chemicals to kill bacteriophage that contaminate dairy systems. They also found that lytic bacteriophage were more resistant to temperature than lysogenic/temperate bacteriophage. Only the bacteriophage isolated against *A. faecalis* (G23) were resistant to high (60°C) temperatures in this chapter. Only three of the ten isolated *E. coli* (G25) bacteriophage were still able to grow after exposure to 60°C, whereas all *S. aureus* (G16) bacteriophage were inhibited by this temperature. The ability to resist temperature is linked to the host bacteria which the bacteriophage infect and the environment in which they were derived (Anon, 2009). Bacteriophage isolated in this chapter responded to a range of pHs in previously

documented ways with most bacteriophage remaining viable between pH 5 and 8 but were rapidly inactivated below pH 3 (Anon, 2009). Bacteriophage are noted to be more resistant to chemicals than bacteria (Anon, 2009), however, this could not be tested in this study as chemicals that lysed MFB were removed from the analysis.

4.4.2.5 Bacteriophage PCA Profiles

There were a number of factors which differentiated the response of bacteriophage; trypsin, triton-x and ribonuclease A were responsible for the majority of the clustering observed whereas acidic buffer (pH 2) and response to chloroform were also important. Future studies could therefore test novel bacteriophage with these five reagents to accelerate classification.

Alcaligenes faecalis (G23) bacteriophage all responded similarly to chemicals by clustering together, although they were isolated from geographically distinct areas (Edinburgh and Aberdeen) and different sample types; scab-infected fleece (M7F) and soil from a sheep pen (D1). The isolation of similar bacteriophage from different environment types has been previously observed (Wolf *et al.*, 2003). *E. coli* (G25) bacteriophage were split into two main groups which correlated to the environmental sample type, whether a liquid or solid environmental sample.

All three *S. aureus* (G16) bacteriophage were isolated from sheep fleece, which confirms observations of *S. aureus* isolated from sheep fleece in Chapter 2. Two distinct bacteriophage identified by their plaque morphology (Table 4.6), which also did not cluster together based on their profiles were isolated from the same sample W6. This demonstrates that more than one bacteriophage may be present in each environmental sample. This analysis aids in the differentiation of bacteriophage isolates, so that distinct bacteriophage can be chosen for bacteriophage infection/experimental work.

4.4.2.6 Effect of Bacteriophage on MFB in Liquid Culture

All MFB produced satisfactory standard curves under laboratory conditions. These are important as they calibrate optical density (OD) values specific to each bacterium. Optical density is a simple, repeatable and quick method for measuring bacterial growth. The conversion from OD to CFU/ml, however, is specific both to the bacterium and the specific laboratory conditions used.

It was important to investigate the effect of isolated bacteriophage both in solid and liquid phases. This is to mimic the spatial structure of the internal gut cavity of mites (Abedon & Culler, 2007), which may have both solid and liquid niches.

Two different effects on bacterial growth were observed with *S. aureus* (G16) bacteriophage in liquid culture. Ph10 inhibited bacteria even at low bacteriophage levels (MOI 0.5) to densities half that of bacteria-only controls. With Ph11 and Ph14, however, bacterial growth appeared to be completely halted within 24 h, compared to sample types and profiles. After this time, however, there was evidence of the emergence of resistant bacterial clones. This is often seen in bacteriophage/bacteria interactions (K Stanley *pers. comm.*). This was not observed in the bacteriophage cocktail (a mixture of Ph10, 11 & 14) which reduced *S. aureus* growth to half that of the bacteria-only control, or with the *E.coli*-bacteriophage cocktail. The emergence of bacteriophage-resistant bacteria may occur by spontaneous mutation independent of the bacteriophage or as an acquired response (Lea & Coulson, 1949). This production of bacteriophage insensitive mutants (BIMs) may be caused by the loss, modification or masking of bacteriophage receptors on the cell wall (Anon, 2009). For this reason bacteriophage cocktails are often used to overcome this potential resistance (Weld *et al.*, 2004) as observed in this study.

Alcaligenes faecalis (G23) bacteriophage were not very effective at killing the bacterium (*A. faecalis*) in liquid culture, although some bacteriophage treatments (Ph15) did significantly lower the OD within 48 h. There was also evidence of a dose-dependent response to bacteriophage with MOI 0.5 Ph16 producing a significantly less efficient effect on the bacterium than Ph16 MOI of 1.

E. coli (G25) bacteriophage appeared to inhibit the growth of the bacteria for the first 6 h, by which point the bacteria-only control was increasing. After this point, however, bacterial growth started to emerge in the bacteriophage treatments, with a range of antimicrobial effects seen with the different bacteriophage isolates. The most effective was Ph9 which significantly reduced the OD by 48 h compared to the bacteria-only control. The *E. coli* bacteriophage cocktail, as seen with the *A. faecalis* cocktail, produced an intermediary effect on bacteria growth. Although not as effective as some published experiments, such as O'Flynn *et al* (2004) who observed complete clearance of viable *E. coli* cells after bacteriophage treatment, the bacteriophage used in this chapter did reduce

bacterial growth compared to the bacteria-only control. Optical density may have been affected by other components in the mixture such as bacterial debris or bacteriophage particles (Abedon, 2008).

There are a number of factors that could affect the bacteriophage infection cycle:

The mechanism of bacteriophage lysis of the bacteria. Although bacteriophage of these three MFB use the holin-endolysin mechanism for lysis (Loessner *et al.*, 1998; Young *et al.*, 2000), *S. aureus* (G16) is a Gram negative bacteria, lacking the additional outer membrane of the other two Gram negative bacteria (*A. faecalis* G23; *E. coli* G25). This may affect the bacterium response to bacteriophage.

The burst size of each bacteriophage may affect its antimicrobial power. This is the number of bacteriophage virions released during each round of lytic growth (Abedon, 2008). It is also known that different factors such as the presence of calcium ions, temperature, pH, physiological state of the sensitive bacteria cells, may affect absorption stage of bacteriophage (Capra *et al.*, 2004) in addition to latent period and initial bacteriophage dose (Weld *et al.*, 2004). Future work should investigate this if bacteriophage are chosen for biocontrol.

These experiments tested single bacterial cultures, yet the internal cavity of mites harbour a community of bacteria (Chapter 2). Therefore the effect of bacteriophage on mixed bacterial cultures, possibly with the utilisation of real-time PCR would be needed to elucidate the effect of bacteriophage on whole community dynamics. Moreover this thesis has not tested the effect of bacteriophage on biofilms, which *E. coli* (G25) has been shown to form (Doolittle *et al.*, 1996). Biofilms are surface-associated communities, which are encased in extracellular polymeric substance (EPS) matrix in a highly heterogeneous structure (Lu & Collins, 2007). Biofilms grow on the walls of vessels/organisms and are often resistant to antimicrobials (Sillankorva *et al.*, 2008a). There is the potential that bacteria within *P. ovis* exist in this way.

4.4.3 Effect of Bacteriophage on *P. ovis* Survival

4.4.3.1 Toxicity of Bacteriophage Buffer to *P. ovis* Mites

There was no evidence of bacteriophage buffer being directly toxic to *P. ovis* mites. This indicated that it was safe to be fed to mites to observe bacteriophage effects on internal bacteria.

When mites were administered one of four different bacteriophage in lamb serum, there was a significant reduction in survival for all treatments compared to the controls. There were different levels of effect observed, with Ph8 (infective for *E. coli* G25) being consistently effective at reducing mite survival rate over the experimental time. Ph14, however, (infective against *S. aureus* G16) appeared to lose efficacy over time. Overall, the two bacteriophage infective against *E. coli* had a greater negative impact on mite survival than *S. aureus* bacteriophage. This could indicate a closer interaction between *E. coli* and mites compared to *S. aureus*. The observed negative effects of bacteriophage on mite survival may have been due to the mites reducing feeding activity owing to the presence of bacteriophage.

4.4.3.2 Effect of Bacteriophage on Bacterial Density within Mites

A higher bacterial density was observed in dead mites compared to live mites after administration of bacteriophage. This was converse to when antibiotics were administered to mites (Chapter 3). This could indicate a mechanism of mite death being facilitated by bacteriophage attacking specific bacteria which then allows the proliferation of other bacteria, usually kept in control by the targeted bacteria (Harcombe & Bull, 2005) and it is therefore the proliferation of other bacteria that ultimately kills the mite through changes in internal microbial dynamics. The prevention of colonisation by pathogens as a result of gut commensal bacteria is known as competitive exclusion (Lan *et al.*, 2005). In addition, the bacterial density of lamb serum controls (live and dead) was significantly lower than all bacteriophage treatments, except for Ph9 but the reason for this is unclear. Bacteriophage lysates were prepared and assumed to be pure, free of any contaminating bacteria, but this cannot be excluded as a source of additional bacteria observed in the bacteriophage-administered mites.

4.4.3.3 Recovery of Bacteriophage

Mites that had been administered bacteriophage were crushed and plated onto bacteriophage-specific bacteria to recover any bacteriophage present internally within the mite. There was evidence that bacteriophage are naturally present in mites, as bacteriophage infective against *A. faecalis* (G23) was detected at the start of the experiment from a few of the mites. This is not surprising as bacteriophage are ubiquitous in nature and mites may encounter them on the sheep skin surface as bacteriophage have been shown to be present wherever bacteria exist (Skurnik & Strauch, 2006). This particular bacteriophage, however, disappeared within 24 h of administration following introduction of a bacteriophage infective towards a different bacterium. By day five of the experiment, both types of experimentally introduced bacteriophage (against *S. aureus* G16 and *E. coli* G25) were recovered from mites. This result indicated a number of observations. Firstly bacteriophage are able to enter the internal cavities of *P. ovis*, although the route is unknown. Aside from ingestion one possible route is *via* grooming behaviours of the mites (Waldorf, 1978). Moreover bacteriophage have been shown to diffuse through membranes (Bruttin & Brussow, 2005) owing to their size (average 50 nm) (Weinbauer & Rassoulzadegan, 2004). Secondly, the presence of novel introduced bacteriophage appeared to change the internal microbial dynamics of *P. ovis* although further work is required to understand these changes.

Studying the transmission of bacteriophage among mites or on sheep skin would be interesting as it may be *via* faecal bacteria transmission, where bacteria excreted by one mite may be picked up by another individual (Sinclair & Filan, 1989). If bacteria and bacteriophage were present in the *in vitro* chambers used in this study, the bacteriophage could replicate every time it encountered a suitable bacterium so an increasing number of bacteriophage would be present in the chambers over the course of the experiment, which the mites could encounter.

Bacteriophage experiments in liquid (Section 4.3.2.7) showed that bacteriophage could inhibit bacterial growth within 24 h of administration and *in vitro* mite feeding experiments also observed significant effects on mite survival within this time period. Under optimal laboratory conditions bacteriophage have shown to lyse bacteria within 30 min (Ellis & Delbruck, 1939), which could explain why effects were seen within such

short time periods. Moreover, bacterial growth on agar plates was much lower in the bacteriophage treatments than the lamb serum control.

Further work is required to isolate bacteriophage that are infective against potential endosymbionts of *P. ovis* such as the arthropod-symbiont *Comamonas* sp. which was detected in *P. ovis* in Chapter 2. Although this bacterium would need to be cultured before bacteriophage could be isolated, it may be possible to target symbionts with bacteriophage. The only known characterised bacteriophage against a symbiont is *AP*SE-1, which infects the secondary symbiont of the pea aphid (*Acyrthosiphon pisum)* (van der Wilk *et al.*, 1999). Although the effects of this bacteriophage on the aphid are undetermined, aphids deficient of endosymbionts are sterile, growth retarded and have reduced longevity, indicating bacteriophage presence may impact negatively on the aphid's physiology (van der Wilk *et al.*, 1999).

4.4.4 Conclusions

This chapter has investigated the potential of isolating bacteriophage infective against mite-associated bacteria and their effect on the survival of *P. ovis* mites.

The isolation of bacteriophage from scab-infected fleece indicates that bacteriophage are naturally present on sheep. As bacteriophage were also isolated from mites it is unclear of the origin of this bacteriophage, but it does indicate that *P. ovis* may be involved in the proliferation of bacteriophage and transfer to the skin, as shown before with other organisms (Heildebrand & Wolochow, 1962; Bomsel & Alfsen, 2003). *P. ovis* could therefore transmit 'control' bacteriophage to other mites on the skin. Toxicity tests would be required to ensure bacteriophage present on the skin did not harm the sheep, however, there is little evidence of bacteriophage being toxic to mammals (Bruttin & Brussow, 2005). Moreover, if bacteriophage are delivered to the skin, where bacteria are present and cause secondary infection skin lesions, the bacteriophage may also help to treat these infections.

Further tests are required to highlight and assess the impact of fleece components on the viability of bacteriophage. Taylor *et al.,* (2008) noted that wool wax and suint inhibited the growth of entomopathic fungi on sheep skin. With bacteriophage,

however, survival appears to be enhanced by the presence of host proteins (Anon, 2009).

The most effective form of control may be an integrated combination of chemical and biological control (O'Neill, 1997) as seen with bacteriophage therapy and antibiotics (Dabrowska *et al.*, 2005) or potentially even bacteriophage and essential oils. Although bacteriophage biocontrol is now widely investigated (Summers, 2001), this is the first known study to investigate the use of bacteriophage biocontrol for microbial control of arthropods.

4.4.5 Summary

This chapter has shown evidence that lytic bacteriophage infective against three mite-associated bacteria (*S. aureus*, *A. faecalis* and *E. coli*) can be isolated from the environment.

- Isolated bacteriophage were characterised by plaque morphology, bacteriophage type and response to chemicals, temperature and enzymes. The isolated bacteriophage can lyse bacteria in both solid and liquid phases.

- Bacteriophage specific to MFB appear to alter the internal bacterial dynamics of *P. ovis* and reduce the survival time of *P. ovis* mites.

- MFB-specific bacteriophage may not be suitable for biocontrol of *P. ovis*, however, may be suitable as part of a bacteriophage cocktail if specific or beneficial bacteria for *P. ovis* are identified.

- The question still remains as to whether bacteriophage can be used to target intracellular bacteria such as an endosymbiont. There is no knowledge of this use currently which may be due to the difficulties associated with culturing endosymbionts *in vitro,* which is required for bacteriophage isolation.

5 General Discussion

Psoroptes ovis mites are the causative parasites of sheep scab disease. It is a contagious disease which causes intense pruritus, wool loss and the development of lesions, which are exacerbated by secondary bacterial infections. Bacteria appear to play an integrated role in the pathogenicity of this disease and are found in the internal cavities of *P. ovis*. The aim of this study was to investigate the bacterial associations of *P. ovis* and to assess them as microbial targets for the control of sheep scab disease.

P. ovis mites from *in vivo* cultures and natural infections, in addition to sheep fleece (scab-infected and healthy) were analysed. The microbial communities were found to be very complex, with a variety of species and bacterial groups identified. Some bacteria were common to all environments, whereas others were isolated from one sample type.

To investigate the effect of disrupting the internal bacterial communities of *P. ovis* on mite survival*, in vitro* feeding experiments were carried out with *P. ovis* mites in the laboratory. Initially mite chambers were constructed and diets optimised to encourage maintenance of *P. ovis* off-host. Antibiotics were used to target bacteria and experiments revealed that *P. ovis* survival and internal bacterial densities were significantly reduced with the administration of antibiotics.

The study also analysed the potential of bacteriophage for the microbial control of sheep scab disease. Bacteria isolated from *P. ovis* faecal trails were used to isolate bacteriophage from environmental samples. The bacteriophage were then characterised by a number of methods. *In vitro* experiments with bacteriophage were also investigated, resulting in a significantly reduced mite lifespan seen with some bacteriophage lysates.

5.1 Microbial Communities Associated with *P. ovis*

The first objective of the study was to identify the microbial communities associated with *P. ovis* mites and sheep scab-infected fleece. The microbial communities associated with sheep scab disease are very complex; no single bacterium appears to be associated with *P. ovis* and scab-infected fleece (Chapter 2). A number of bacteria isolated from *P. ovis* mites in this study, including *Corynebacterium* and *Propionibacterium acnes* have not been previously isolated from *P. ovis*. There were some genera, such as *Staphylococcus* spp. and *Pseudomonas* spp.*,* which although isolated from *P. ovis* mites in this study, are too ubiquitous in the environment to act as suitable targets for the biocontrol of sheep scab

disease. One limitation to the project was the use of different individual samples for bacterial identification, for example the use of different samples from natural infections used for *P. ovis*-associated bacterial identification (S193, S21, S22) to scab-infected fleece-associated bacterial identification (S9, S14, S23). This was owing to quantity and quality of the sample types, i.e. not all natural infection samples that were received contained *P. ovis* mites, however, comparison of results from individual samples would have generated more powerful results.

RISA analysis of fleece indicated similar bacterial communities were present in scab-infected and healthy fleece yet densities of bacteria in scab-infected fleece were lower than healthy fleece in this study. RISA analysis of *P. ovis* bacterial communities, however, indicated some differences in community compositions due to location and timing of sampling, although there did not appear to be differences in diversity (measured by Shannon-Weaver) between natural infection or *in vivo* cultured mites.

DNA molecular sequencing of the 16S rRNA gene and screening *P. ovis* mites using endosymbiont-specific PCR revealed the presence of the known arthropod symbiont, *Comamonas* sp. (Zouache *et al.*, 2009b). Further research is needed into the prevalence of this bacterium in global populations of *P. ovis* and its relationship with *P. ovis,* to ascertain its importance to this mite. If this bacterium has commensal or even mutualist associations with *P. ovis* it may be a potential target for microbial biocontrol of sheep scab disease. Although there have been a number of studies examining the effect of removing endosymbionts, for example in aphids (Wilkinson, 1998; Hardie & Leckstein, 2007) and ticks (Morimoto *et al.*, 2006), and despite this bacterium being a known arthropod symbiont (Kikuchi *et al.*, 2005a), it has not yet been closely studied in this way in other arthropod hosts.

In addition to bacteria isolated from whole-mite extracts, bacteria which are transient and excreted by the mite were detected in faecal pellets (Chapter 2). *P. ovis* mites are assumed to have a narrow diet (Sinclair & Filan, 1989; Mathieson & Lehane, 1996) yet the diversity of bacteria identified indicates that these bacteria may contribute to the diet of *P. ovis*, making it more complex than first thought. The hypothesis of *P. ovis* harbouring beneficial bacteria is based on the assumption that obligate beneficial bacteria are commonly observed in arthropods living on specialised, and often narrow diets, and have frequently been shown to synthesise essential nutrients and cofactors for

the host (Atlas & Bartha, 1993; Egert *et al.*, 2003; Banjo *et al.*, 2005), such as aphids and *Buchnera* sp. (Baumann, 1997) or tsetse flies and *Wigglesworthia* (Aksoy, 2000).

5.2 *P. ovis* Survival

Following bacterial identification, the relationship between *P. ovis* and internal bacteria on mite survival was investigated. *In vitro* chambers were constructed to assess the impact of bacterial removal on *P. ovis* survival. Although these mites are challenging to maintain in the laboratory, the *in vitro* chambers used in this study allowed the differentiation of effects on mite survival between treatments. Moreover the survival times observed were comparable to previous *in vitro* culturing efforts (Table 3. 1) (O'Brien *et al.*, 1994b; Mathieson, 1995; Smith *et al.*, 1999).

Chapter 2 highlighted the complex community of internal bacteria in *P. ovis*, both internally and excreted by the mite. Two of the mite faecal bacteria (G17, G27) isolated by culture-based techniques were resistant to all five antibiotics (ampicillin, chloramphenicol, gentamicin, penicillin, tetracycline) tested in pure liquid cultures. However when antibiotics (gentamicin, tetracycline) were administered in lamb serum presented to *P. ovis* as food, the mites' survival was significantly reduced compared to the control treatments. There could be more than one bacterial species present that may be important to mite survival and therefore combinations of antibiotics may be necessary to knock out all important bacteria to reduce mite survival dramatically enough for disease control. Targeting of the potential symbiont *Comamonas* sp. directly, perhaps with antibiotics previously shown to have specific bactericidal effects such as fluoroquinolones or trovafloxacin (Abraham & Simon, 2007) is required.

The occurrence of *P. ovis* feeding in the *in vitro* chambers was confirmed using detection of rabbit IgG by ELISA. This sensitivity of this technique could be improved upon in future with the use of large sample numbers. A number of additional techniques were investigated to detect the uptake of serum by the mites. Magnetic beads coated in antibodies were unsuccessful. Increasing the number of (pooled) mites tested may overcome some of the technical problems encountered. To remove the necessity of feeding, microinjection of antibiotics directly into *P. ovis* was also investigated (Section 3.3.4.1), but this proved instantly fatal to the mites.

Although the entry route/mechanism is not known, bacteriophage that matched the administered lysates, were successfully recovered from the internal cavity of mites (Chapter 4), providing evidence for bacteriophage effects internally within the mite. This indicates that bacteriophage may be suitable agents for a novel control of sheep scab disease as they can penetrate *P. ovis*.

To confirm feeding (and ingestion of treatment) in future studies, a potential alternative method to detect and differentiate the intake of serum type (i.e., rabbit or lamb) may be a PCR assay specific for the cytochrome *b* gene. This gene has been used for elucidating phylogenetic relationships of mammals owing to its sequence variation between species and the wealth of data available (Castresana, 2001). Zehner *et al.,* (1998) were able to identify species-specific cytochrome *b* genes through the use of PCR and RFLP patterns when digested with the restriction enzyme *AluI*. This may be useful as the cytochrome *b* gene is localised in the mitochondrial genome and this mtDNA is present in much higher copy numbers than nuclear DNA (Zehner *et al.*, 1998). This technique may therefore be able to detect much lower levels of ingested serum than the ELISA investigated in Chapter 3.

Survival experiments with *P. ovis in vitro* (Chapter 3) indicated that altering the microbial dynamics within *P. ovis* had negative effects on the mites' survival time. Decreased survival has been observed when the aphid symbiont *Buchnera* was selectively removed (Koga *et al.*, 2007). In other arthropod-symbiont relationships, removal of the symbiont has been shown to result in reduced host fecundity (Zhong *et al.*, 2007) or growth (Bandi *et al.*, 1999) which although could be possible in *P. ovis*, it was not possible to measure these variables using the current *in vitro* system.

5.3 Bacteriophage Isolation

Bacteriophage were hypothesised as a novel control of sheep scab disease by targeting internal bacteria associated with *P. ovis* mites. Bacteriophage have a number of advantageous properties over other antimicrobials including self-replication, specificity, environmentally-derived and there is no evidence of toxicity towards animals.

Bacteriophage infective towards *P. ovis*-associated bacteria were isolated from the environment and assessed for their suitability for bacteriophage biocontrol of sheep

scab disease. Chapter 4 provided evidence that sixteen lytic bacteriophage infective against three mite faecal bacteria (*S. aureus*, *A. faecalis* and *E. coli*) were isolated from the environment. This is a promising start towards isolating a bacteriophage specific for microbial control of *P. ovis*. The isolated bacteriophage could lyse bacteria in both solid and liquid phases. All bacteriophage that were isolated from the environment were characterised by plaque morphology, bacteriophage type (lytic/lysogenic) and response to chemicals, temperature and enzymes. The results of these tests were then used to differentiate isolates. Further sampling is required to isolate bacteriophage infective against the remaining mite faecal bacteria isolated in this study to investigate their potential for *P. ovis* control.

When administered to *P. ovis* in lamb serum, bacteriophage appeared to alter the internal bacterial dynamics of *P. ovis* compared to the serum-only controls and significantly reduced the survival time of *P. ovis* mites. Converse to the hypothesis that administration of bacteriophage would reduce bacterial density, mites fed bacteriophage showed an increase in bacterial density (from log 5 to log 8). Furthermore, mites that were alive when sampled had a much lower bacterial density than mites which died during the experiment. This could indicate a mechanism of death, as introduction of bacteriophage disrupted the normal balance of bacterial flora within *P. ovis*, resulting in a proliferation of potentially pathogenic, opportunistic bacteria. There is a wealth of literature on the effects of modifying bacterial community structures. Ecological networks are extremely fragile to selective attacks (Solé & Montoya, 2001) such as when targeted by antibiotics and can have large effects on community stability. The disruption may also be correlated with the organism changing from a healthy to susceptible state (Alonzo *et al.*, 2011). The increase in bacterial density observed after administration of bacteriophage to mites in Chapter 4 may be an example of competitive exclusion (Lan *et al.*, 2005) as the removal of naturally present flora by bacteriophage allows the proliferation of opportunistic bacteria, which may be pathogenic to the mite. Ingestion of bacteria can also induce cell death and tissue damage, as seen in *Drosophila* (Buchon *et al.*, 2009). Ecological specialisation due to symbionts has been observed in pea aphids (Ferrari *et al.*, 2007) and a reduced performance of the aphid was seen when the symbiont was removed and other bacteria were introduced. Opportunistic pathogenic bacteria may be 'mediators of killing' such as the pigment phenazine, which is produced by *Pseudomonas aeruginosa* and

has been shown to cause mortality in the model nematode *Caenorhabditis elegans* (Mahajan-Miklos *et al.*, 1999). In fish, a number of bacterial pathogens have been identified, including *Aeromonas*, *Pseudomonas*, *Pasteurella* and *Vibrio*, (Thune *et al.*, 1993) two of which (*Pseudomonas*, *Vibrio*) were identified as being present in *P. ovis* in Chapter 2. There was also evidence of bacteriophage proliferation within the mites as plaque size increased over the experimental time (86 h), which is advantageous for biocontrol application.

This study was not able to address the question as to whether bacteriophage can be used to target intracellular bacteria such as an intracellular endosymbiont. There is no knowledge of this use currently which may be due to the difficulties associated with culturing endosymbionts *in vitro,* a prerequisite for bacteriophage isolation. Furthermore, bacteria identified in this thesis have the ability to grow in biofilms, such as *E. coli* and *Pseudomonas* spp. (Tait *et al.*, 2002). Biofilms are "surface-associated communities encased in a hydrated extracellular polymeric substance (EPS) matrix composed of polysaccharide, proteins, nucleic acids and lipids to maintain a heterogeneous structure" (Lu & Collins, 2007). Biofilms are resistant to a number of antimicrobial agents (Sillankorva *et al.*, 2008b) yet bacteriophage have been proposed to treat them (Lu & Collins, 2007), with promising effects observed against *Pseudomoas aeruginosa* (Sillankorva *et al.*, 2008b) and *Enterobacter* spp. (Tait *et al.*, 2002).

Toxicity testing of the bacteriophage would be required before bacteriophage could be used on sheep (Chapter 4, Section 4.1.2) yet the isolation of bacteriophage directly from sheep fleece in this study indicates that bacteriophage are natural constituents of the normal fleece flora. Moreover, bacteriophage have been observed to enter the bloodstream of animals, without any negative side effects (Weber-Dabrowska *et al.*, 1987). These indicate that bacteriophage could be a safe alternative (for both sheep and humans) to current organophosphate chemicals currently used to treat sheep scab disease. Before commercial use bacteriophage chosen for biocontrol would need to undergo testing of their ecology, genomics, behaviour in experimental animals, toxicity/side effects, presence of virulence genes and behaviour in cocktails (Bruttin & Brussow, 2005).

5.3.1 Potential of Bacteriophage for control of *P. ovis*

An objective of this study was to analyse the potential of using bacteriophage to mediate biocontrol of sheep scab mite control. The experimental results have indicated that bacteriophage biocontrol could have potential for this use for a number of reasons:

Bacteriophage in this study (Chapter 4) were effective bactericides of MFB in both solid and liquid phases. It is possible therefore to produce the bacteriophage in a liquid emulsion to be applied as a dipping product. This would have the advantages of sheep dipping, such as bathing of skin wounds/displacement of air from skin without the disadvantages of current dips, such as chemical resistance, human health or disposal (See Chapter 1, Section 1.2.2). Alternatively, the bacteriophage-product could be poured directly onto the sheep or freeze-dried into tablets for storage. Bacteriophage can be produced economically on a large scale, usually in fermenters (Branston *et al.*, 2011).

Chapter 4 started to investigate the infection dynamics of isolated bacteriophage infective against mite faecal bacteria (MFB), which vary depending on the specific bacteriophage/bacteria combination. Biocontrol of sheep scab disease using bacteriophage may be more effective depending on the treatment approach used, whether passive or active. Passive treatment involves flooding of the infection with bacteriophage so the bacterial population is destroyed by the initial lysis of bacterial cells by proliferating bacteriophage. In contrast, active treatment administers a small quantity of bacteriophage and it is the proliferation and transmission of bacteriophage virions to other bacterial cells which controls the infection (Anon, 2009). The timing of application for treatment is important, if the bacteriophage-product was administered prior to *P. ovis* infestation the bacteriophage may die from lack of bacterial hosts or may lose viability in response to environmental conditions. If the bacteriophage-product was administered after a prolonged *P. ovis* infection the disease may be too established and sheep health condition deteriorated beyond treatment. A preventative (prophylactic) would be most desirable to prevent the disease establishing within a flock and causing harm to sheep welfare. But, bacteriophage may not be suitable for use in this way due to potential off-host longevity issues.

This study was not able to investigate the effect of bacteriophage *in vivo* (on scab-infected sheep). This would be necessary before such a control could be commercialised

as Weld *et al* (2004) noted that some bacteriophage changed from behaving as lytic *in vitro* to lysogenic when *in vivo*, which would render the biocontrol product useless.

To analyse the potential for bacteriophage as a biocontrol for sheep scab mites a number of factors should be included. Two isolated bacteriophage (Ph8, Ph9) (Chapter 4) will be compared. These are both infective against *E. coli* and both had significant negative effects on *P. ovis* survival when administered in lamb serum (Chapter 4). Although *E. coli* is not specific to mites, results in Chapter 4 indicated that modification of the internal bacterial communities' composition could have negative effects on *P. ovis* survival.

Ph8 was isolated from a liquid environmental sample (water from a drinking trough), has a small (1 mm) plaque size and was not inhibited by any of the chemical, enzyme or temperature treatments. Ph9, however, which was isolated from sheep faeces, had a much larger plaque size (6 mm), is tailed and was inhibited by all chemical treatments. Although they both had negative effects on *P. ovis* survival the effect on bacterial density appeared to be different, with mites that died after administration with Ph8 having a much higher bacterial density than live mites fed the same bacteriophage lysate. Ph9-fed mites, however, showed the opposite effect, which indicates that the properties of different bacteriophage clearly differ and it is important that these are characterised in detail if they are to be used in a commercial control.

5.4 Conclusion

In conclusion, this thesis investigated the potential of targeting beneficial bacteria in sheep scab mites for biocontrol of sheep scab disease. The bacteria associated with *P. ovis* were characterised and results indicated that *P. ovis* mites do not appear to harbour a single obligate endosymbiont. It was then investigated whether the removal of a fraction of this bacterial population through antibiotic or bacteriophage administration affected optimum mite survival. Further research is required to investigate the effects of knocking out specific bacteria identified in this study, such as *Comamonas* sp., a previously described endosymbiont in arthropods. Bacteriophage infective towards mite-associated bacteria were isolated and it was demonstrated that bacteriophage could be used to reduce bacterial populations in the mite and reduce mite survival rates, in addition to evidence of bacteriophage penetrating *P. ovis* internally. Despite this enormous potential, however, the complexities of *P. ovis* bacterial communities make

bacteriophage biocontrol unsuitable for this application at this stage as a bacterial species critical to *P. ovis* survival was not identified.

As there is still a critical need for an alternative control method for sheep scab disease, the following points remain for future research in this field:

A constant challenge to this project was the availability and quality/quantity of *P. ovis* samples, especially those sourced from natural infections. Therefore further exploration is required to produce a satisfactory *in vitro* system to maintain and ideally culture *P. ovis* off-host to allow for testing of novel control agents. Moreover, further investigation is required into the feeding behaviour of *P. ovis in vitro* for this purpose.

For product application purposes, the diversity of bacteria associated with *P. ovis* from a wider geographical area, as well as different development stages of *P. ovis* mites, should be identified. For example, to investigate the presence and incidence of the known arthropod symbiont, *Comamonas* sp. detected in this study.

If beneficial bacteria are identified, the mechanism of interaction between the bacteria and *P. ovis* should be elucidated and may highlight other methods of biocontrol outwith knocking out the bacteria such as disrupting nutrient supply pathways or disrupting synthetic pathways in the associated bacteria.

Finally, the isolation of bacteriophage against additional *P. ovis* associated bacteria is possible due to the ubiquitous nature of bacteriophage.

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7 Appendices

Appendix 1

Bacterial Identification Tests

Standard microbiological tests, as described below, were carried out on each colony isolated from sheep fleece (scab-infected and healthy) (Section 2.2.2.4) and *P. ovis* faecal trails (Section 2.2.2.5) to aid putative identification of bacteria (Cowan *et al.*, 2003; Whitewolf, 2008) On the basis of these results, bacteria were identified to a group level using a bacterial diagnostic table (Cowan *et al.*, 2003) (Table A1.1).

Microbiological Identification methods

The following biochemical tests were carried out on pure isolates of bacteria to aid identification (Cowan *et al.*, 2003; Whitewolf, 2008).

1. Form & elevation: Bacterial colonies were recorded as related to common form and elevation shapes.
2. Gram stain: This was performed using the KOH string test (Arthi *et al.*, 2003). Briefly, a colony of bacteria was mixed with 3% potassium hydroxide (KOH) on a glass slide for 60 s. The bacterium was Gram negative if stringing occurred when loop was gently pulled out of the emulsion.
3. Morphology: A loopful of bacterium was mixed into a drop of sterile water on a glass slide, allowed to dry then heat fixed. It was then stained using Basic fuschin. After 10 min the excess dye was rinsed off using water. Cells were then examined under 40x and 100x (with immersion oil) lenses.
4. Catalase test: 5 µl of 3% hydrogen peroxide (H_2O_2) was dropped onto a single bacterial colony. Result was positive for the activity of the enzyme catalase if bubbles formed.
5. Oxidase test: A drop of oxidase reagent (Biomérieux) was placed onto filter paper. A loopful of bacterium was then streaked across it. If a colour change to purple was seen within 10 s, test was positive. If purple colour was seen within 60 s, a delayed positive was seen. If purple colour only developed after 60 s, test was negative.

6. Anaerobic growth: Bacteria were streaked onto NA and placed into a chamber with an AnaeroGen sachet (Oxoid). Plates were incubated at 27°C ±2 for 24-48 h.
7. Bacteria that were identified as Gram negative rods were also tested using the API 20E strips (Biomérieux).

Some additional tests were required for a few bacteria samples, which included:

8. Growth at 42°C: Bacteria were streaked onto agar plates, placed into a bronze jar and incubated at 42°C in a water bath for 24 h.
9. Motility: Bacteria were stabbed into a glass tube containing 5 ml of 3% sloppy agar (Oxoid). It was then incubated at 34-37°C for 24 h.

Table A1.1 Bacterial Identification Table (Cowan 2003). Table used to classify bacterial isolates from sheep fleece (healthy and scab-infected) and P. *ovis* faecal trails.

gram reaction	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
shape	coccus (clusters)	coccus (clusters)	coccus (chains)	coccus (tetrads)	rod	rod	irreg. rod	rod	rod	rod	rod	rod	rod	rod	rod	coccus (pairs)
aerobic growth	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
anaerobic growth	–	+	+	+	+	–	–	+	+	–	–	–	+	+	+	–
endospores	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
motility (Motility Medium)	–	–	–	–	–	+	–	+ or –	+ or –	+ or –	+ or –	+ or –	–	+	+	–
catalase reaction	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+	+
oxidase reaction	+	–	–	–	–	–	–	–	+ or –	+ or –	+	+	–	–	+	+
Glucose O/F Medium											–	O	F	F	F	O
Micrococcus	X															
Staphylococcus		X														
Streptococcus			X													
Lactococcus			X													
Enterococcus			X													
Leuconostoc			X													
Pediococcus			X	X												
Aerococcus				X												
Lactobacillus					X											
Kurthia						X										
Arthrobacter							X									
Clostridium								X								
Bacillus									X	X						
Alcaligenes											X					
Pseudomonas												X				
Klebsiella													X			
Shigella													X			
Salmonella														X		
Escherichia														X		
most other enteric genera														X		
Aeromonas															X	
Chromobacterium															X	
Neisseria																X

Appendix 2

Table A2.1 PCR components for bacterial group specific PCR assays (Section 2.2.2.7).

Group	Examples	Reference	Annealing temp (°C)	$MgCl_2$ (mM)	dNTPs (µM)	primer conc (µM)
General Bacterial		Fierer *et al* 2005	53	1.5	0.2	0.3
Firmicutes	*Bacillus, Staphylococcus*	Fierer *et al* 2005	65	1.5	0.2	0.3
Acidobacteria*	*Acidobacter capsulatum*	Fierer *et al* 2005	50	1.5	0.2	0.3
Actinobacteria	*Actinomyces*	Stach *et al* 2003	72	1.5	0.2	0.3
Bacteroidetes	*Bacteroides*	Fierer *et al* 2005	67	2.0	0.2	0.3
α-Proteobacteria	*Acetobacter aceti*	Fierer *et al* 2005	60	2.0	0.2	0.3
β-Proteobacteria*	*Spirillum serpens*	Fierer *et al* 2005	60/63	1.5/2.0	0.2	0.3
γ-proteobacteria	*Acinetobacter psychrotolerans*	Vanbroekhoven *et al* 2004	58	1.5/2.0	0.2	0.3
γ-proteobacteria	*E.coli*	Sabat *et al* 2000	72	2.0	0.2	0.3
γ-proteobacteria	*Pseudomonas*	Widmer *et al* 1998	65	1.5	0.2	0.3
γ-proteobacteria	Erwinia-Pba 28	Heuser *et al* 2003	53	2.0	0.2	0.3/0.5/1.0
γ-proteobacteria	*Salmonella typhimurium*	Salehi *et al* 2005	60	2.0	0.2	0.4

Table A2.2 Thermal cycling conditions for bacterial group specific PCR assays (Section 2.2.2.7).

Group	Examples	Annealing temp °C	Initial	Cycles	Temperature profile	Extension
General Bacterial		53	5 min 94°C	30	60 s 94°C, 30 s 53°C, 60 s 72°C	2 min 72°C
Firmicutes	*Bacillus, Staphylococcus*	65	5 min 94°C	30	60 s 94°C, 30s 65°C, 60 s 72 °C	2 min 72°C
Acidobacteria*	*Acidobacter capsulatum*	50	5 min 94°C	30	60 s 94°C, 30 s 50°C, 60 s 72°C	2 min 72°C
Actinobacteria	*Actinomyces*	72	4 min 95°C	35	30 s 95°C, 60 s 72°C, 60 s 70°C	2 min 72°C
Bacteroidetes	*Bacteroides*	58	15 s 95°C	50	30 s 95°C, 10 s 58°C, 30 s 74°C	2 min 74°C
α-Proteobacteria	*Acetobacter aceti*	60	5 min 94°C	30	60 s 94°C, 30 s 60°C, 60 s 72°C	2 min 72°C
β-Proteobacteria*	*Spirillum serpens*	60	5 min 94°C	30	60 s 94°C, 30 s 60°C, 60 s 72°C	2 min 72°C
γ-proteobacteria	*Acinetobacter psychrotolerans*	67	5 min 94°C	30	60 s 94°C, 30 s 67°C, 60 s 72°C	2 min 72°C
γ-proteobacteria	*E.coli*	72	4 min 94°C	30	45 s 94°C, 45 s 72°C,	10 min 72°C
γ-proteobacteria	*Pseudomonas*	65	5 min 94°C	40	30 s 94°C, 60 s 65°C, 60 s 72°C	10 min 72°C
γ-proteobacteria	*Erwinia*	53	2 min 94°C	30	30 s 94°C, 60 s 53°C, 60 s 72°C	2 min 74°C
γ-proteobacteria	*Salmonella typhimurium*	64	60 s 95°C	35	60 s 95°C, 30 s 64°C, 30 s 72°C	2 min 74°C

Appendix 3

Table A3.1 PCR components for endosymbiont bacteria PCR assays (Section 2.2.2.7).

	Endosymbiont PCR Components (Primers)			
	Cardinium **Ch-F/ Ch-R**	***Comamonas*** ***Com199F/ Com614R***	***Wolbachia*** **81F/ 691F**	***Rickettsia*** **EHR16sD/ EHR16SR**
Reaction volume	25 µl	25 µl	25 µl	25 µl
Forward primer	0.25 µM	0.5 µM	0.25 µM	0.20 µM
Reverse primer	0.25 µM	0.5 µM	0.25 µM	0.20 µM
MgCl2	2 mM	2.5 mM	2.5 mM	2 mM
dNTPs	200 µM	200 µM	200 µM	200 µM
Buffer	1 x	1 x	1 x	1 x
Hot start GoTaq (Promega)	1.25 U	1.25 U	1.25 U	1.25 U
DNA	2 µl	2 µl	2 µl	2 µl

Table A3.2 Thermal cycling conditions for endosymbiont-PCR assays (Section 2.2.2.7).

Endosymbiont	**PCR cycling conditions**			
Cardinium	2 min 94°C	30 x	30 s 92 °C, 30 s 57°C, 30 s 72°C	5 min 72°C
Comamonas	2 min 94°C	40 x	15 s 94 °C, 60 s 63°C, 30 s 72°C	7 min 72°C
Wolbachia	2 min 94°C	35 x	60 s 94 °C, 60 s 55°C, 60 s 72°C	4 min 72°C
Rickettsia	2 min 94°C	40 x	30 s 94 °C, 30 s 55°C, 45 s 72°C	5 min 72°C

Appendix 4

Table A4.1 Results of the biochemical tests for bacteria isolated from healthy and scab-infected fleece. KOH: Potassium hydroxide string test (Arthi *et al.,* 2003),Gram: Gram reaction, catalase and oxidase: presence of these enzymes, shape: morphology of colony by staining and microscopy. (1) Biochemical classification as Cowan (2003). Table continued on page 243 and 244.

Disease status	Sample	Agar	Colony morphology	KOH	GRAM	Catalase	Oxidase	Shape	Biochemical Classification[1]
	F1	Nutrient	white minute	-	+	+	-	cocci	*Staphylococcus*
	F1	serum	glossy white	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	F1	serum	glossy Y/W	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	F1	serum	glossy yellow	+	-	+	+	cocci	*Neisseria/Branhamella*
	F1	Mac	pink	-	+	+	-	rod?	*Kurthia*
	F1	Mac	pale pink	-	+	+	-	cocci	*Staphylococcus*
Healthy	F1	Mac	red purple	-	+	+	-	cocci	*Staphylococcus*
	F1	MRS	cream/yellow	+	-	+	-	cocci	*Acinetobacter*
	F1	MRS	glossy yellow	-	+	-	-	cocci	*Streptococci*
	F1	MRS	spread white	-	+	+	-	rod	*Kurthia*
	F1	Pseud	white circular	-	+	+	-	cocci	*Staphylococcus*
	F1	Pseud	creamy/gloopy	-	+	+	-	rod	*Kurthia*
	F1	Pseud	minute colonies	-	+	+	-	cocci	*Staphylococcus*
	F24	PSEUD	white circular	-	+	+	-	cocci	*Staphylococcus*
	F24	PSEUD	white minute	-	+	+	-	cocci	*Staphylococcus*
	F24	PSEUD	small furry	-	+	+	+	cocci	*Micrococcus*
	F24	MRS	minute yellow	-	+	-	-	cocci	*Streptococci*
	F24	MAC	red/purple	-	+	+	-	cocci	*Staphylococcus*
Healthy	F24	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F24	serum	golden yellow	-	+	-	-	cocci	*Streptococci*
	F24	serum	white	-	+	-	-	cocci	*Streptococci*
	F24	Nutrient	white	-	+	-	-	cocci	*Streptococci*
	F24	Nutrient	cream yellow	-	+	+	-	cocci	*Staphylococcus*
	F24	Nutrient	yellow/white	-	+	+	-	cocci	*Staphylococcus*
	F38	Nutrient	golden yellow	-	+	+	-	cocci	*Staphylococcus*
	F38	Nutrient	white	-	+	+	+	cocci	*Micrococcus*
	F38	Nutrient	white/yellow	-	+	+	-	cocci	*Staphylococcus*
	F38	Nutrient	cream	-	+	+	-	cocci	*Staphylococcus*
	F38	Nutrient	white/yellow	-	+	+	+	cocci	*Micrococcus*
Healthy	F38	serum	cream	+	-	+	+	short rod	*Unidentified*
	F38	Mac	pink	-	+	+	-	cocci	*Staphylococcus*
	F38	Mac	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F38	Mac	colourless	-	+	+	+	rod	*Bacillus*
	F38	MRS	gloopy	-	+	+	+	rod	*Bacillus*
	F38	Pseud	minute cream	-	+	+	+	cocci	*Micrococcus*
	F41	Nutrient	golden yellow	-	+	+	-	cocci	*Staphylococcus*
	F41	Nutrient	white circular	-	+	+	-	rod	*Kurthia*
	F41	serum	yellow	+	-	+	+	cocci	*Neisseria/Branhamella*
	F41	serum	white circular	+	-	+	+	cocci	*Neisseria/Branhamella*
	F41	MRS	small cream circula	-	+	+	-	cocci	*Staphylococcus*
Healthy	F41	MRS	minute cream	+	-	+	-	cocci (L)	*Acinetobacter*
	F41	Mac	bright pink	-	+	+	-	cocci	*Staphylococcus*
	F41	Mac	orange-pink	-	+	+	-	cocci	*Staphylococcus*
	F41	Mac	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F41	Pseud	yellow	-	+	+	-	cocci	*Staphylococcus*
	F41	Pseud	white/cream	-	+	+	-	cocci	*Staphylococcus*
	F60	Nutrient	white	-	+	+	-	cocci	*Staphylococcus*
	F60	Nutrient	gloopy	-	+	+	-	rod	*Kurthia*
	F60	Nutrient	white filiform/lobate	-	+	+	-	cocci	*Staphylococcus*
	F60	serum	bright yellow	+	-	+	-	cocci	*Acinetobacter*
	F60	serum	golden yellow	-	+	+	+	rod	*Bacillus*
	F60	serum	white	-	+	+	-	cocci	*Staphylococcus*
Healthy	F60	serum	yellow	+	-	+	+	cocci	*Neisseria/Branhamella*
	F60	Mac	orange-pink	+	-	+	-	cocci	*Acinetobacter*
	F60	Mac	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F60	MRS	minute cream	+	-	+	-	cocci	*Acinetobacter*
	F60	MRS	minute cream	+	-	+	-	cocci	*Acinetobacter*
	F60	Pseud	minute cream	-	+	+	-	cocci	*Staphylococcus*
	F60	Pseud	yellow	-	+	+	-	cocci	*Staphylococcus*
	F60	Pseud	yellow	-	+	+	-	rod	*Kurthia*

Disease status	Sample	Agar	Colony morphology	KOH	GRAM	Catalase	Oxidase	Shape	Biochemical Classification[1]
	F70	PSEUD	white filamentous	-	+	+	-	cocci	*Staphylococcus*
	F70	PSEUD	yellow circular	-	+	+	-	cocci	*Staphylococcus*
	F70	Nutrient	yellow circular	-	+	+	+	cocci	*Micrococcus*
	F70	Nutrient	white minute	-	+	+	+	cocci	*Micrococcus*
	F70	serum	yellow circular	-	+	-	-	cocci	*Streptococci*
Healthy	F70	MRS	white glossy	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	F70	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F70	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F70	MAC	bright red	-	+	+	-	cocci	*Staphylococcus*
	F70	MAC	red/purple	-	+	+	-	cocci	*Staphylococcus*
	F70	serum	white/cr circular	-	+	+	+	cocci	*Micrococcus*
	F70	Nutrient	white/yellow circ	-	+	+	+	cocci	*Micrococcus*
	F88	Nutrient	cream	-	+	+	-	cocci	*Staphylococcus*
	F88	Nutrient	yellow	+	-	+	-	cocci	*Acinetobacter*
	F88	MAC	pale pink	+	-	+	-	cocci	*Acinetobacter*
	F88	Pseud	bright yellow	-	+	+	+	spirulus	*Unidentified*
Healthy	F88	Pseud	gloopy white	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	F88	MRS	gloopy	-	+	+	+	rod	*Bacillus*
	F88	Pseud	minute cream	-	+	+	+	cocci	*Micrococcus*
	F88	Nutrient	cream	+	-	+	+	cocci	*Neisseria/Branhamella*
	F88	serum	cream	+	-	+	+	short rod	*Unidentified*
	F91	Nutrient	white	-	+	+	-	cocci	*Staphylococcus*
	F91	Nutrient	cream	-	+	+	-	cocci	*Staphylococcus*
	F91	Nutrient	white	-	+	+	-	cocci	*Staphylococcus*
	F91	serum	glossy white	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	F91	serum	white	-	+	+	+	cocci	*Micrococcus*
	F91	serum	yellow	-	+	+	+	cocci	*Micrococcus*
Healthy	F91	MRS	minute cream	-	+	+	+	cocci	*Micrococcus*
	F91	MRS	white/yellow glossy	+	-	+	+	cocci	*Neisseria/Branhamella*
	F91	Mac	bright pink	-	+	+	-	cocci	*Staphylococcus*
	F91	Mac	pale pink	-	+	+	-	short rod	*Kurthia*
	F91	Mac	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F91	Mac	pale pink glossy	-	+	+	-	cocci	*Staphylococcus*
	F91	Pseud	creamy	-	+	+	-	cocci	*Staphylococcus*
	F109	Nutrient	white	-	+	+	-	cocci	*Staphylococcus*
	F109	Nutrient	yellow	+	-	+	-	short rod	*Unidentified*
	F109	Nutrient	golden yellow	+	-	+	+	cocci	*Neisseria/Branhamella*
	F109	Nutrient	white liquidy	-	+	+	+	rod	*Bacillus*
	F109	Nutrient	white	-	+	+	+	rod	*Bacillus*
	F109	MAC	purple	-	+	+	+	cocci	*Micrococcus*
Healthy	F109	MAC	pale pink	+	-	+	+	short rod	*Unidentified*
	F109	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
	F109	MRS	small cream	-	+	+	-	cocci	*Staphylococcus*
	F109	Pseud	liquidy white	+	-	+	+	short rod	*Unidentified*
	F109	Pseud	cream/white	-	+	+	+	cocci	*Micrococcus*
	F109	serum	cream/white	+	-	+	+	short rod	*Unidentified*
	F109	serum	cream	+	-	+	+	cocci	*Neisseria/Branhamella*
	F109	serum	golden yellow	-	+	+	-	cocci	*Staphylococcus*
	FA	PSEUD	cream circular	-	+	+	+	cocci	*Micrococcus*
	FA	PSEUD	yellow circular	-	+	-	-	cocci	*Streptococci*
	FA	MRS	minute	-	+	+	-	cocci	*Staphylococcus*
	FA	MRS	white fluffy	-	+	+	+	cocci	*Micrococcus*
	FA	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
Healthy	FA	MAC	purple	-	+	+	-	cocci	*Staphylococcus*
	FA	serum	cr/yellow circular	-	+	+	-	cocci	*Staphylococcus*
	FA	serum	golden yellow	-	+	+	-	cocci	*Staphylococcus*
	FA	serum	cream glossy	-	+	+	-	cocci	*Staphylococcus*
	FA	Nutrient	cream	-	+	+	-	cocci	*Staphylococcus*
	FA	Nutrient	golden yellow	-	+	-	-	cocci	*Streptococci*

Disease status	Sample	Agar	Colony morphology	KOH	GRAM	Catalase	Oxidase	Shape	Biochemical Classification[1]
	S1	serum	cream circular	-	+	-	-	cocci	*Unidentified*
	S1	serum	white circular	-	+	+	+	cocci	*Micrococcus*
	S1	serum	bright yellow	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S1	Nutrient	cream circular	-	+	-	-	cocci	*Streptococci*
	S1	Nutrient	cream circular	-	+	+	-	cocci	*Staphylococcus*
Scab-infected	S1	Nutrient	glossy	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S1	MRS	golden yellow	-	+	-	-	cocci	*Streptococci*
	S1	MRS	yellow	-	+	-	-	cocci	*Streptococci*
	S1	PSEUD	yellow circular	-	+	-	-	cocci	*Streptococci*
	S1	PSEUD	white circular	-	+	+	+	cocci	*Micrococcus*
	S1	MAC	dark pink	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S1	MAC	pale pink	-	+	+	-	cocci	*Staphylococcus*
	S2	PSEUD	yellow circular	-	+	+	-	cocci	*Staphylococcus*
	S2	PSEUD	margin	-	+	+	+	rod	*Bacillus*
	S2	serum	filamentous	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	serum	circular	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	serum	filamentous	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
Scab-infected	S2	Nutrient	filamentous	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	Nutrient	white globular	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	Nutrient	dark pink	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	MRS	fluffy white	-	+	+	-	cocci	*Staphylococcus*
	S2	MAC	dark red	+	-	+	+	rod	*Alcaligenes/Pseudomonas*
	S2	MAC	bright pink	+	-	+	-	cocci/rod	*Unidentified*
	S2	Nutrient	Bright yellow	+	-	+	+	rod	*Alcaligenes/Pseudomonas*

Appendix 5.

Table A5.1 Proportion of each major bacterial group in healthy (a) and scab-infected fleece samples (b). Peterson's homogeneity index (PHI= 1-(0.5*(sum ai-bi))) was calculated (=1.00). A PHI can be between 0-1, where zero indicates homogeneous populations and 1.00 indicates heterogeneous populations.

Major Bacterial Group	Mean Healthy (a)	Mean Scab-Infected (b)	Healthy-Infected (ai-bi)
Staphylococcus	0.46	0.17	0.28
Streptococci	0.07	0.18	-0.11
Micrococcus	0.14	0.09	0.05
Pseudom/Alicaligenes	0.04	0.47	-0.43
Bacillus	0.05	0.04	0.01
Acinetobacter	0.07	0.00	0.07
Kurthia/Corynebacterium	0.06	0.00	0.06
Neisseria/Branhamella	0.06	0.00	0.06
Unidentified	0.06	0.04	0.02
Peterson's Heterogeneity Index			**1.00**

Appendix 6

Table A6.1 Results of biochemical tests on bacteria isolated from *P. ovis* faecal trails. Biochemical classification as Cowan (2003).

Sample	Agar	Gram	Colour	Catalase	Oxidase	Morphology	Anaerobic growth	Biochemical Classification
G16	Nutrient	+	cream	+	-	cocci	y	*Staphylococcus*
G17	Nutrient	-	white	+	+	cocci	y	*Neisseria*
G18	Pseudomomas	+	yellow	+	w+	cocci	y	*Unidentified*
G19	Pseudomomas	+	orange	+	w+	cocci	y	*Micrococcus*
G22	Serum	-	white minute	+	+	rod	y	*Unidentified*
G23	Serum	-	yellow/orange	+	+	cocci	y	*Alcaligenes*
G24	Serum	+	bright yellow	+	+	cocci	y	*Micrococcus*
G25	Serum	-	cream glossy	+	-	rod	y	*Escherichia*
G26	Mac	+	white glossy	-	-	rod	y	*Bacillus*
G27	Mac	-	yellow	+	-	cocci	n	*Unidentified*

Appendix 7.

Table A7.1 Potential functions of bacteria identified from *P. ovis* mites in this study. Continued on page 246.

Phylum	Bacteria species	Host	Reference	Important functions[a]
Actinobacteria	*Corynebacterium* spp	sheep abscess	Tadayon *et al* 1980	Non-motile rods, aerobic to microaerophilic, widely distributed in nature, causes disease by exotoxins.
	Propionibacterium acnes	*P. ovis (midgut)*	Hogg & Lehane 2001, Hamilton *et al* 2003)	Rods, non-motile, gram positive. Excellent anaerobic growth, but able to grow aerobically. Originally isolated from acne pustules (Bisset 1962). Produces propionic acid from carbohydrates. Animal surfaces have a high lipid content. Lipase action on sebum triglycerides (Nanazi 2007).
Firmicutes	*Bacillus cereus*	Healthy fleece & sheep fleece rot	Lyness *et al* 1994, Kloos *et al* 1976, Chin & Watts 1992	Rods, form spores, motile, gram positive. Aerobic, facultative anaerobic growth. Widely distributed in soil and nature. Carry out polymerative cleavage of sucrose to fructose and glucose.
	Bacillus thuringiensis	Healthy fleece, gut of caterpillars	Lyness *et al* 1994	Pathogenic variety of *B cereus* . Causes of death of larvae in certain insects.
	Bacillus spp	*P. ovis(midgut)* & healthy fleece	Oliveira *et al* 2006, Murray & Edwards 1987, Chin & Watts 1992, Hogg & Lehane 2001, Hamilton et al 2003.	
	Staphylococcus aureus	Healthy fleece & sheep fleece rot	Oliveira *et al* 2006, Chin & Watts 1992	
	Staphylococcus intermedius, S. chromogenes	*P. ovis*	Hogg & Lehane 1999	Cell wall contains organic phosphate, ribitol, glucosamine, muramic acid, glycine, lysine, aspartic acid, serine, glutamic acid, alanine, (sm amounts of) threonine, proline, valine, leucine. Proteases, lipases, phospholipidases, lipoprotein, esterases lysases produced (toxins which enhances invasiveness of bacteria in tissue). Most strains will hydrolyse native animal proteins e.g haemoglobin, fibrin, gelatin, releasing fatty acids. Can invade tissues to become pathogenic.
	Staphylococcus hyicus	unknown	Meyer *et al* 2001	
	Staphylococcus xylosus	Healthy fleece	Lyness *et al* 1994, Kloos *et al* 1976	
	Staphylococcus sp	*P. ovis* mite, healthy fleece, infected lice	Kloos et al 1976, Murray & Edwards 1987, Chin & Watts 1992, Hogg & Lehane 1999, Hogg & Lehane 2001,	

Table A7.1 continued.

Phylum	Bacteria species	Origin	Reference	Important functions[a]
Beta-proteobacteria	*Comamonas* sp	Asian tiger mosquito *(Aedes albopictus)*	Zouache *et al* 2009	Potential symbiont (Zouache *et al* 2009)
Gamma-proteobacteria	*Acinetobacter* spp.	sheep fleece rot (skin surface)	Chin & Watts 1992, Zouache *et al* 2009, Hamilton *et al* 2003	Haemolytic activity. (*A iwoffii* phenotypically similar to *Moraxella osloensis*).
	Pseudomonas stutzeri	*D. ovis* lice & sheep fleece rot	London & Griffith 1984, Murray & Edwards 1987, Zouache *et al* 2009	Observed in fleece rot and *D. ovis* (Murray & Edwards 1987).
	Pseudomonas putida	Healthy fleece & sheep fleece rot	London & Griffith 1984, Lyness *et al* 1994	Common soil inhabitants.
	Pseudomonas spp.	Healthy fleece	Lyness *et al* 1994, Hogg & Lehane 2001,	Versatile, biochemically active, proteolytic.
	unid pseudomonoads	*P. ovis*	Hogg & Lehane 2001	
Unknown	*uncult bacterium*	Wood boring beetle (*Anoplophora glabripennis*)	Geib *et al* 2009	Digestion

Appendix 8.

Table A8.1 Bacteria identified in this study with the number of *rrn* operons. * Summarised from Lee *et al* (2009) unless otherwise stated.

Phylum	Bacteria	Number of operons	Reference*
Actinobacteria	*Corynebacterium amycolatum*		
	Corynebacterium diphtheriae	5	
	Micrococcus luteus	2	
	Nocardia beijingensis		
	Propionibacterium acnes	3	
	Rathayibacter tritici		
	Tropheryma whippeli	1	
	Bacteroides fragilis	6	
Firmicutes	*Bacillus sp*		
	Bacillus cereus	10	Klappenbach *et al* 2000
	Bacillus fusiformis		
	Bacillus thuringiensis	13-14	
	Carnobacterium mobile		
	Staphylococcus sp		
	Staphylococcus aureus	6-9	Lee *et al* 2009, Gürtler & Barrie 1995
	Staphylococcus chromogenes		
	Staphylococcus epidermidis, AF269309.1	6	
	Staphylococcus hyicus		
	Staphylococcus xylosus		
Beta-Proteobacteria	*Alcaligenes faecalis*		
	Comamonas testoreroni	3	
Gamma-Proteobacteria	*Acinetobacter genomosp*		
	Acinetobacter iwoffii	6	
	Escherichia coli	7	Morgan *et al* 1977
	Klebsiella oxytoca		
	Moraxella osloensis		
	Pseudomonas sp		
	Pseudomonas chlororaphis		
	Pseudomonas mendocina		
	Pseudomonas putida	6	Klappenbach *et al* 2000

Appendix 9

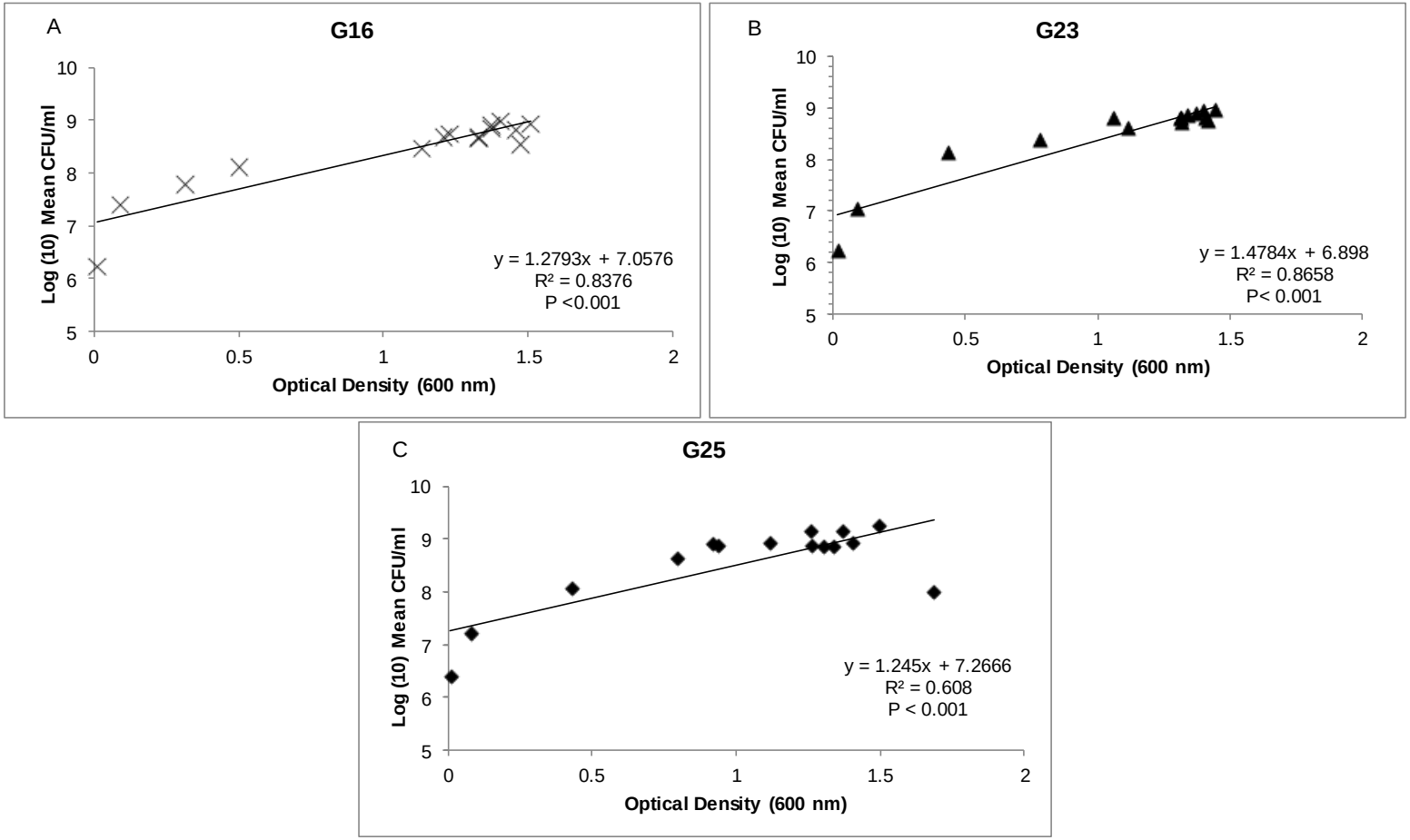

Figure A9.1 MFB standard curves for optical density and CFU/ml. Log_{10} mean CFU/ml versus optical density (600nm) for MFB *S. aureus* (G16; A), *A. faecalis* (G23; B) and *E. coli* (G25; C). Regression equations given on each figure.

Appendix 10

Standard Curve of Antibiotic Effect on Bacteria

Specific assay organisms are used to test the affects of certain antibiotics (Brock, 1970). Mite reference bacteria that most closely matched the assay organisms were used to measure the ZOI (mm) at each antibiotic concentration (μg/ml) to produce a standard curve of effect (Ratcliff *et al.*, 1981). A range of concentrations of each antibiotic was used: 0.5, 1, 2.5, 5, 12.5, 25, 50, 100 and 200 μg/ml. The aim was to produce a reference that could quantify the amount of antibiotic within a mite by spot lysis assays.

Results: Standard Curve for Antibiotics

Standard curves for each antibiotic were produced from optical density (OD 600 nm) and colony forming units (CFU/ml) results. These equations can then be used to back-calculate antibiotic-intake from mites. (Table A9.1).

Table A10.1 Standard curves for ZOI (mm) and antibiotic concentration (μg/ml) for five antibiotics measured with reference bacteria on NA. Antibiotic concentration = a*ZOI (mm) + b.

Bacteria	Antibiotic	R^2	a	b
G16	Tetracycline	0.97	0.345	0.623
G18	Penicillin	0.77	0.799	-0.214
G24	Gentamicin	0.78	0.574	-0.12
G24	Ampicillin	0.61	0.508	-0.066
G26	Chloroamphenicol	0.83	0.623	-0.028